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THE UNIVERSITY OF ALBERTA

INVESTIGATIONS ON THE EFFECTS OF ETHYLENE  
ON MITOCHONDRIAL SYSTEMS

by

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A THESIS

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The undersigned certify that they have read,  
and recommend to the Faculty of Graduate Studies for  
acceptance, a thesis entitled "Investigations on the  
Effects of Ethylene on Mitochondrial Systems" submitted  
by Arthur Olaf Olson in partial fulfilment of the  
requirements for the degree of Doctor of Philosophy.





## ABSTRACT

The effect of ethylene on plant tissues has been the subject of extensive research since the turn of the century. Since the major response to ethylene seems to involve changes in respiratory rates, a study of the effect of ethylene on mitochondria prepared from a variety of tissues was conducted.

Ethylene was found to increase the rate of mitochondrial volume changes induced by adenosine diphosphate and adenosine triphosphate. As well, the gas appeared to increase the rate of mitochondrial adenosine triphosphate hydrolysis. However, the gas had no effect on partially purified adenosine triphosphatases prepared from bean cotyledon and rat liver mitochondria.

The effects of increased adenosine triphosphatase activity were simulated by a digital computer, and it was demonstrated that a fairly small change in the concentration of adenosine diphosphate was sufficient to greatly modify mitochondrial respiratory characteristics, and hence overall cell metabolism. Such a change in the concentration of adenosine diphosphate could result from the effect of ethylene on adenosine triphosphate hydrolysis, and is within normal ranges of concentration of adenosine diphosphate.



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## LIST OF ABBREVIATIONS

ATP	:	Adenosine triphosphate
ADP	:	Adenosine diphosphate
NAD	:	Nicotinamide adenine dinucleotide
NADH	:	Nicotinamide adenine dinucleotide (reduced)
Pi	:	Inorganic phosphate
ANS	:	8-anilino-naphthalene sulfonic acid
DNP	:	2,4-dinitrophenol
BSA	:	Bovine serum albumin
Mw	:	Washed mitochondrial suspension
-SH	:	Sulfhydryl group
-S-S-	:	Disulfide bond
RCR	:	Respiratory control ratio (Ratio of oxygen uptake in state 3 to that in state 4)
ADP/O	:	Ratio of the number of atoms of ADP phosphorylated to the number of oxygen atoms taken up.
ppm	:	Parts per million
IBB	:	Isobutanol-benzene reagent



## INTRODUCTION

The gas, ethylene, has been shown to affect a large variety of plant processes, and has often been called a plant hormone (Pentzer, and Heinze 1954, Ulrich 1958, Burg 1962, Spencer 1965). As well, it is an anaesthetic for mammals, though its use has decreased because of the explosive nature of ethylene-oxygen mixtures.

Some of the effects ethylene may induce are stimulation of fruit ripening (Sievers, and True 1912, Chace, and Denny 1924, Harvey 1926, Davies and Church 1931, Wolfe 1931, Gane 1937, Hansen 1939, 1943, Smock 1942, Pratt, and Biale 1944, Heinze, and Craft 1953, Biale 1960, Rakatin, and Povolotskaya 1961, Workman, and Pratt 1957, Varner 1961, Biale, and Young 1962, Burg 1962, Spencer 1965), an increase in the rate of chlorophyll degradation (Denny 1924a, Chace, and Church 1927, Davies, and Church 1931, Zimmerman, and Hitchcock 1931, Crocker, Hitchcock, and Zimmerman 1935, Vogeles 1937, Miller, Winston, and Schumer 1941, Rohrbough 1943, Nickerson 1948, Hansen 1955), inducement of epinastic responses in seedlings (Doubt 1917, Crocker, Hitchcock, and





Zimmerman 1935, Denny and Miller 1935, Denny 1935, 1938, 1939, Zimmerman and Hitchcock 1933, Kidd and West 1945), abscission of leaves (Zimmerman and Hitchcock 1933, Elmer 1937, Kidd and West 1945, Livingstone 1950, Jacobs 1955, Heck, Pires, and Hall 1961, Rosen, and Siegel 1963), production of drowsiness in flowers (Crocker, and Knight 1908, Darley, Duggar, Mudd, Ordin, Taylor, and Stephens 1963), prevention of cell elongation (Hansen, and Rose 1915, Denny 1926, Zimmerman, and Hitchcock 1931, 1933, Crocker, Hitchcock, and Zimmerman 1935, Ulrich 1958), initiation of adventitious roots (Zimmerman, and Hitchcock 1933, Zimmerman, Crocker, and Hitchcock 1933, Zimmerman, Hitchcock, and Crocker 1931), inducement of swelling in roots (Hansen, and Rose 1915), stimulation of isocoumarin production in carrots (Carlton, Peterson, and Tolbert 1961), breaking of dormancy (Haber 1926, Vacha, and Harvey 1927, Elmer 1932, Hitchcock, Crocker, and Zimmerman 1932, Mack, and Livingstone 1933, Denny, and Miller 1935), changes in membrane permeability (Gaumann, and Jaag 1947, Roberts 1951, Young, Bieleski, and Biale 1961, Rowan, McGlasson, and Pratt 1961, Sacher 1962, Baur, and Workman 1964, Lyons, and Pratt 1964, Brown, Jackson, and Dupuy 1964), flower induction (Rodrigues 1932),





reversal of tropisms (Reinhert 1959), causal agent in plant diseases (Walker 1957, Williamson 1950, Sadasivan 1961, Millerd and Scott 1962, Darley et al 1963), blanching of celery (Mack 1927), softening of pecan shucks (Finch 1936), increase in the rate of carbon dioxide production (Harvey 1913, Hitchcock, Crocker, and Zimmerman 1932, Heinze, and Craft 1953, Grierson, and Newhall 1955), inhibition of the growth of adventitious roots (Crocker, Hitchcock, and Zimmerman 1935, Zimmerman, and Wilcoxon 1935), and inhibition of the growth of cotton (Hall, Truchelot, Leinweber, and Herrero 1957, Heck, Pires, and Hall 1961).

#### Characteristics of ripening tissue

The most important commercial application of ethylene at present is the induction of fruit ripening (Spencer 1965). The general process of ripening is characterized by a change in coloration, primarily caused by a loss of chlorophyll (Crocker et al 1935, Davies, and Church 1931, Vogele 1937), a softening of tissues, where the main effect is the formation of more soluble forms of protopectin (Davies, and Church 1931, Hansen 1939, 1943, Biale 1960, Biale, and Young 1962), and a change in the rate of respiration.



Fruits which show a gradual decline in their rate of respiration, followed by a sudden increase in respiration during the ripening process, are called climacteric fruits. This increase in respiration may be induced by ethylene (Denny 1924b, Gane 1935, Hansen, and Hartman 1937, Biale, Young, and Olmstead 1954, Pratt, and Workman 1962, Burg, and Burg 1962). There is no abrupt change in the respiration of non-climacteric fruit. However, their respiration may be stimulated at any time if the fruit is detached, and this stimulation is proportional to the amount of ethylene added (Pratt, and Workman 1962).

Ethylene also causes acceleration of carbon dioxide production in other parts of the plant (Heinze, and Craft 1953, Livingstone 1950, Roberts 1951, Grierson, and Newhall 1955, Hackett 1959).

As well, there are changes in soluble sugar levels (Biale, and Young 1962), and in mitochondrial content. During the final stages of ripening, and the beginning of senescence, there appears to be a decrease in the amount of mitochondria present (Buchloch 1960, Varner 1961). Lysis, or destruction of mitochondria may occur. Tedeschi, and Harris (1958), and others (Lehninger 1964) have shown that mitochondria behave as osmometers





hence may be broken by osmotic changes occurring in the ripening tissue. There may be a fusion to form larger organelles, or a decrease in their rate of synthesis. This latter point assumes that there is a dynamic equilibrium present with continuous synthesis and degradation. Akazawa and Beevers (1957) followed the increase and then decrease of the mitochondrial nitrogen content in germinating castor bean endosperm, and suggested that such a continuous turnover occurs. A number of other workers have found a similar increase in mitochondrial content in germinating seeds (Opik 1965, Bain and Mercer 1966, Cherry 1963, Hansen, Vatter, Fisher, and Bils 1959, Howell 1961, Young, Huang, Vanecko, Marks, and Varner 1960). Lund, Vatter, and Harrison (1958) demonstrated a progressive increase in the number of mitochondrial cristae during maturation of corn root tip cells, indicating a possible increase in mitochondrial enzyme content.

It would thus seem that the mitochondrion becomes more complex with time. At a certain point, some stimulus appears to cause its breakdown, with a concomitant decrease in overall cell respiration. Hansen, Vatter, Fisher and Bils (1959) found that



mitochondria prepared from germinating corn scutellum increased in respiratory activity up to the fourth day, when respiration began to decline. Opik (1965) found a similiar, though more rapid, cessation of respiratory activity in mitochondria prepared from bean cotyledons.

### Theories regarding ethylene's mode of action

Evidence for the biochemical action of ethylene in initiating ripening, senescence, or any of the other effects of ethylene is quite meagre. A certain minimal concentration of ethylene must be present in a fruit before the process of ripening can be initiated, and the fruit must have reached a particular physiological state if it is to respond to this concentration of gas (Rohrbough 1943, Nickerson 1948, Hansen 1955, Burg and Burg 1962, 1965). Ripening can be forestalled by high carbon dioxide, or low ethylene concentrations. The latter condition is produced under vacuum, or low oxygen concentrations (Biale 1960, Burg and Burg 1965, Young, Romani, and Biale 1962).





## Uncoupling of Oxidative Phosphorylation

Millerd, Bonner, and Biale (1953) first suggested that ethylene affected plant tissue, particularly fruits, by uncoupling the oxidative processes from phosphorylation. They had found that the rate of respiration of the pre-climacteric, but not of the climacteric avocado fruit, was limited by the capacity of the phosphate transfer system. 2,4-dinitrophenol (DNP), which uncouples oxidative phosphorylation (Lehninger 1960, Racker 1961, Borst, and Slater 1961), seemed to have little effect on climacteric fruit. Pearson, and Robertson (1952) found that DNP increased the respiration rate of pre-climacteric fruit, but not of post-climacteric fruit. They suggested that at a critical stage in development, the increasing demands for protein synthesis in larger cells may result in an increase in concentration of phosphate acceptors. These acceptors, by taking phosphates more rapidly from the intermediates of the respiratory system, would cause an increase in the rate of respiration.

Spencer (1959) found that DNP initiated an increase in carbon dioxide production, and a decrease in



ethylene evolution in mature green tomatoes, with no effect upon other stages of maturity. Hulme, Jones, and Wooltorton (1963) demonstrated a small decrease in the efficiency of oxidative phosphorylation at post-climacteric stages as related to pre-climacteric stages. This was corroborated by Eisenhardt, and Rosenthal (1964), who presented evidence that post-climacteric fruit were still capable of oxidative phosphorylation. Marks, Bernlohr, and Varner (1957) were able to show that oxidative phosphorylation reached a maximum in the early climacteric, and maintained that level until senescence was fairly well advanced.

Rowan, Robertson, and Pratt (1957), and Rowan, Pratt, and Robertson (1958) have shown that the so-called "high-energy" phosphate compound production parallels carbon dioxide production even during the climacteric peak, and that a net synthesis of ATP was the result. There was very little change in the concentration of ADP. This indicates that the respiratory efficiency is quite good, with coupling between the oxidative process and the synthesis of ATP. Hence it is unlikely that the level of phosphate acceptor regulates the response to ethylene, though of





course, both the levels of phosphate, and phosphate acceptor would be rate controlling in respiration rate increases. Evidence presented by Rowan, and his co-workers (1957, 1958), and by McGlasson, and Pratt (1964), who found that the rate of phosphate incorporation increased during the climacteric, bears out the presence of such a control system.

Thus, uncoupling of oxidative phosphorylation does not appear to be the cause of ripening. Uncoupling may however occur in the late post-climacteric stages, when polyphenols (Lieberman, and Biale 1956), or an enzyme system such as an adenosine triphosphatase, may become active (Millerd, Bonner, and Biale 1953). The former case is certainly a possibility, for in many fruits, the content of polyphenols and tannins increases with age (Roberts 1942).

The effect of ethylene upon membrane permeability

One of the more commonly noted changes in ripening fruit is the softening of the tissue. Kidd, and West (1938), and Blackman, and Parija (1928), suggested that a permeability change was the cause of the climacteric rise in respiration. Sacher (1962) found





a very good correlation between the increase in rate of leakage of cell contents, and the climacteric rise. As well, he was able to show an increase in amino acid incorporation during the senescent stages (Sacher 1966). In relation to this thesis, Sacher (1959) was able to demonstrate that permeability changes occur during senescence of non-climacteric material such as bean endocarp.

Such a loss of selective permeability could be expected to alter protoplasmic compartmentalization, thus affecting equilibria and inhibitions between various enzymes and substrates. This could cause a change in the rate of respiration, as well as the other metabolic changes associated with the climacteric. The decline in respiration following the climacteric maximum could be attributed to further cellular disorganization as a result of the change in membrane permeability. For instance, reaction of cytoplasmic oxidases with mitochondrial phenolic compounds, could produce polymerized quinones (Roberts 1942), which are uncouplers of oxidative phosphorylation (Lieberman, and Biale 1956). Similarly, Abdul-Baki, McCollum, and Dickinson (1965) found that tomatoes were less able to metabolize malate and pyruvate as they ripened, even



though the ratio of moles of phosphate to moles of oxygen incorporated (P/O) remained constant. This would suggest a decrease in enzyme availability, or an inhibition.

Young, and his co-workers (1961) observed that climacteric avocado tissue lost phosphate ( $P^{32}$ ) more readily than preclimacteric tissue. Baur, and Workman (1964) found that the rate of ion leakage from banana tissue increased up till two days before the onset of the climacteric, when it reached a constant rate. This latter point is also interesting, in that it negates the proposition of Burg, Burg, and Marks (1964) that ethylene did not alter the loss of water from bananas, or pea slices, but that the increase in leakage of sugars, as shown by Sacher (1962), is simply due to an increase in endogenous levels of the sugars.

Roberts (1951) demonstrated that ethylene first decreased, then increased the rate of water uptake by germinating wheat. This is possibly a result of modification of the seed coat permeability.

Brown, Jackson, and Dupuy (1964) were able to show that ethylene increased the rate of glucose





leakage in onions. This effect was not inhibited by DNP, so appears to be a purely physical effect, with little or no energy requirement.

Van Fleet (1963) found that low concentrations of ethylene were sufficient to cause changes in the pattern of distribution of enzymes involved in tissue differentiation, and development.

Sacher (1966) has indicated that free space in bananas increased significantly about forty-four hours before, and rose exponentially during the climacteric. The increase in free space indicates a progressive increase in the proportion of cells which become freely permeable to solutes in the ambient solution by simple diffusion. Other workers have found a similar increase in free space in the avocado during ripening (Ben-Yehoshua 1964).

Lyons, and Pratt (1964) found that ethylene treatment of mitochondrial systems prepared from both animal and plant tissue caused an increase in the rate of mitochondrial swelling. As the mitochondrial membrane is a factor in regulation of cell metabolism, the importance of this observation is apparent. The control over respiration is exerted by limiting the rate of movement of ADP, and ATP (Lehninger 1964), and may



explain the triggering action of ethylene in fruit ripening.

The oxidation of certain compounds, such as  $\alpha$ -ketoglutaric acid, can be augmented by the addition of the supernatant fractions to preparations of sub-cellular particles from climacteric, but not pre-climacteric fruits (Romani, and Biale 1957). One interpretation of this result is that changes occur during the climacteric that make the mitochondrion more permeable to components in the soluble phase of the cell.

Chandra, and Spencer (1962, 1963) have shown that ethylene evolution increases as mitochondria are aged in vitro. This might indicate that activation of enzymes necessary for ethylene production did not occur until the mitochondrion is breached. On the other hand, it could indicate that ageing, or disintegration of mitochondria under natural conditions results from ethylene accumulation, and that the burst in ethylene evolution is caused by increased substrate availability. Regardless of where the ethylene is formed in the cell, if ethylene causes mitochondrial swelling observed by Lyons et al (1964),





and that seen in electron micrographs of pear tissue during ripening (Bain, and Mercer 1966), an increase in respiration would result. Crane (1961) has suggested that such an increase in respiration could be a result of the greater surface area of the mitochondrion, hence increase in enzyme availability, and membrane permeability.

Of course, the effect on membrane permeability need not be only on mitochondria. Most likely, such a response would be shown by all lipid containing membranes in the cell. For instance, modifications in the vacuolar membrane could alter substrate availability to the mitochondrion.

McGlasson, and Pratt (1964), in comparing methods for determination of ethylene in tissues, found that an evacuation method gave higher values than did sampling of internal spaces in the tissue, indicating that ethylene is present primarily in a dissolved, or adsorbed form. Ethylene is about fourteen times as soluble in lipid as in water, and considering that the main locations of lipid in the cell are the various membrane systems, this lends further support to ethylene altering membrane permeability (Eger, and



Larson 1964). Currier, and Peoples (1954) suggested that the toxic properties of light hydrocarbons could be correlated to their distribution coefficients between oil and water, or oil and air. It is possible that the effect of ethylene could be a result of accumulation of the gas in lipid layers in the cell. Van Fleet (1963), using fairly high concentrations of ethylene, was able to show changes in lipoprotein distribution and average unsaturation. However, the concentrations of ethylene used were extremely non-physiological, and the analytical methods open to question.

Work on various anaesthetics has shown that the solubility of the gas in lipids is the major factor that differentiates one anaesthetic from another. There is a close correlation between oil-air partition coefficients, and anaesthetic potency (Eger, and Larson 1964).

It is interesting to note that gamma irradiation of lemons stimulates increased carbon dioxide and ethylene production (Maxie, Eaks, Sommer, Rae, and El-Batal 1965). Sufficient ethylene was produced to cause degreening of the fruits, and a respiration rate resembling that of climacteric fruits, though atypical





for citrus fruits (Maxie, Eaks, Sommer, Rae, and El-Batal 1965, Denny 1924b).

A similar effect is shown with avocado (Young 1965), where gamma irradiation can induce increased respiration and ethylene production only in preclimateric fruit. Irradiation of climateric fruit had no effect on the rate of respiration, or on ethylene formation.

Treatment of pea epicotyls with gamma irradiation causes an increase in ethylene evolution in amounts not proportional to dosage (Shah, and Maxie 1965). It is possible that the radiation affects the cellular membranes, inducing a change in permeability resulting in stimulation of metabolic pathways producing ethylene.

#### The auxin-ethylene theory of action

Many processes that are controlled by auxin are also influenced by ethylene (Crocker, Hitchcock, and Zimmerman 1935, Hall, and Morgan 1963). Similarly, certain effects induced by ethylene such as the increase in size of cortical cells (Isaacs 1938, Richards, and MacDougal 1904), and tomato leaf epinasty,



attributed to an acceleration of growth on the basal upper side of the petiole (Turkova 1942), are suggestive of an increase in auxin concentration.

Ethylene does not affect the pea test, or the avena curvature test (Marinos 1960). Hence inactivation or slowing of auxin transport does not appear probable, at least at the levels of auxin, and the conditions used in these experiments. Long term treatment (several days) does however inhibit the polar transport of auxin (Morgan, and Gausman 1966). Short term treatments (three hours) have no effect (Abeles 1966).

Burg, and Burg (1966a, 1966b) have presented evidence that induction of flowering in the pineapple is controlled by ethylene, rather than auxin. 3-indole-acetic acid is presently used for this purpose, though ethylene was once used. Ethylene also aids the rooting of cuttings, both independently, and synergistically with auxin (Michener 1935). Other workers have shown ethylene to enhance the sensitivity of growing cells to auxin (Michener 1938, Marinos 1960).

The effect of ethylene may be to increase cell permeability, hence rate of entry of auxin. The reverse may also be true, for 3-indole-acetic acid





appears to act in part by altering the structure of the primary cell wall, or of the middle lamellar substance, thus resulting in an increase in permeability (Heyn 1940, Cleland, and Bonner 1956).

Several of the auxins appear to accelerate ethylene production by a tissue, hence some of the responses noted earlier may actually be the result of the high levels of ethylene present (Zimmerman, and Wilcoxon 1935, Morgan, and Hall 1964, Abeles, and Rubinstein 1964). A similar response in ethylene evolution has been shown after treatment with 2,4-dichlorophenoxyacetic acid (2,4-D) (Yamaki 1947, Morgan, and Hall 1962, Hansen 1946).

Using a variety of inhibitors, Abeles (1966) has suggested that the mechanism of action of growth regulators on the enhancement of ethylene production is the induction of enzymes involved in the biogenesis of ethylene.

The auxin-ethylene balance has been advanced as the control mechanism in abscission (Williamson 1950, Gawadi, and Avery 1950, Hall 1952). Hall, and Morgan (1963) reported that fairly high levels of ethylene (100 - 1000 parts per million) stimulated the activity of the 3-indole-acetic acid oxidase system in cotton.





This interaction could result in a direct change in the balance, and explain Michener's response (Michener 1938).

Thus, there appears to be a relationship between ethylene and auxin. This would suggest that both act upon the same general location.

#### The effect of ethylene on various enzymes

Ethylene may also affect various enzyme systems directly by either increasing production of an enzyme through induction, or by removal of inhibitions. For instance, removal of permeability barriers could occur.

The induction of an enzyme through the action of ethylene is possible, however, would not account for the very rapid response to ethylene.

Van Fleet (1963) found that saturated solutions, and solutions containing 1000 parts per million of ethylene, induced certain changes in lipoproteins, and appeared to activate proteolytic enzymes. An increase in concentrations of malic enzyme, and pyruvate carboxylase were found in apples treated with ethylene by Hulme, and his co-workers (Hulme, Jones, and Wooltorton 1963, 1965, Jones, Hulme, and Wooltorton 1965). Rakatin (1946) stated that the use of ethylene



in the ripening of fruits increased the activity of carboxylase, and cocarboxylase. Tager, and Biale (1957) found no carboxylase or aldolase activity in the preclimacteric fruit pulp. Both enzymes made their appearance during the climacteric, and increased in activity until several days after the climacteric peak had been reached. Barker, and Solomos (1962) gave evidence for an increase in the rate of turnover, and concentration of fructose diphosphate in apples during the climacteric, apparently caused by an activation of, or a removal of barriers to phospho-fructokinase. A similar effect was found with bananas.

Mitochondrial preparations from climacteric tomatoes are less able to metabolize malate, and pyruvate than earlier stages of the fruit, even though P/O ratios were maintained (Abdul-Baki, McCollum, and Dickinson 1965). This would indicate a decrease in synthesis of these acids, or a compartmentalization resulting in a lower availability.

Ethylene may stimulate the enzymes involved in degradation and softening of pectin during the climacteric, resulting in general softening of the tissue (Hansen, and Elmer 1937, Davies and Church 1931,







Hansen 1939, 1943, Biale 1960, Biale, and Young 1962, Spencer 1965). This appears to be caused primarily by the activation of pectin methyl esterase, which degrades protopectin to the more soluble forms of pectin, and pectic acid.

Roberts (1951) found no major disturbances in protein hydrolysis in germinating wheat treated with ethylene. Hulme (1937, 1948, 1951, 1954a, 1954b), however, found a small increase in the protein content of apples on stimulation by ethylene into the climacteric rise in respiration. Similar results were also shown for avocado, and tomato fruit (Rowan, Pratt, and Robertson 1958).

Many of these effects, such as increases, or decreases in enzyme activity could be related to changes in substrate availability, hence changes in membrane permeability.

Burg, and Burg (1965) suggested that ethylene may affect metal containing enzymes, such as cytochrome c oxidase, because of the marked resemblance between the biological activity of ethylene, and closely related compounds, and their ability to bind to metals. As well, they have presented evidence that oxygen and



ethylene attach to the same metal containing enzyme, and that carbon dioxide is a competitive inhibitor with ethylene for its site on the enzyme. It is interesting to note that analogs of ethylene, and acetylene react with cobalt<sup>III</sup>, a form of cobalt found in vitamin B<sub>12</sub>, however, the extent of this reaction is not known with ethylene at low concentrations (Griffith, and Wilkinson 1959). No further analysis of this possible reaction of ethylene has been done.

#### The effect of ethylene upon germinating seeds

Ethylene decreases the water uptake of germinating wheat seeds during the second to fifth day of germination, and then causes a large increase in water uptake on the fifth day (Roberts 1951). This behavior is consonant with an initial decrease, then an increase in cell permeability. Hale, and Schwimmer (1943) found that ethylene treatment of high moisture wheat decreased heating of the wheat, resulting in a better grade, and a higher percentage of germination. As well, the treatment increased carbon dioxide evolution from the stored wheat.





Since Meheriuk, and Spencer (1964), and Spencer, and Olson (1965) have shown that ethylene is a product of seed metabolism, it is possible that ethylene has a role in seed dormancy, and germination.

Etiolated pea seedlings respond to as little as 0.025 parts per million of ethylene with the so-called "triple response" (Knight, Rose, and Crocker 1910, Crocker, and Zimmerman 1932, Pratt, and Biale 1944). The triple response consists of leaf epinasty, stem swelling and an inhibition of extensive growth.

Vacha, and Harvey (1927) found that ethylene was effective in causing germination of seeds of buck thorn, high bush cranberry, honeysuckle, and snowberry. Since the barrier to germination in these seeds appears to be that of water uptake, ethylene may affect cell or seed coat permeability.

#### Ethylene and disease

Ethylene treatment of citrus fruits induces a type of stem end decay, if excessive levels (1000 to 5000 parts per million) are used (Grierson, and Newhall 1950). In this laboratory, during a preliminary study with Phaseolus vulgaris variety Kinghorn Wax, ethylene





treatment induced a stem rot when very high levels of the gas were used. No organism was tested for in either of the above examples.

The treatment of oranges with ethylene was shown by Loucks, and Hopkins (1946) to increase the amount of Diplodia sp. but not Phomopsis sp. decay. One explanation lies in direct stimulation of the growth of Diplodia. It is also possible that ethylene induced a change in the orange tissue resulting in greater availability of substrates specifically required by Diplodia, but non-essential for Phomopsis.

Ethylene treatment does not always increase a tissues susceptibility to disease, for Stahman, Clare, and Woodbury (1966) found that treatment of sweet potatoes infected with Ceratocystis fimbriata induced an increased resistance to the pathogen, as well as an increase in peroxidase, and polyphenoloxidase activity. As ethylene was detected above the lesions in approximately the same concentrations as used in the treatment, it is possible that ethylene is normally active in creating resistance. The increase in enzyme activity may reflect a change in permeability.



### Ethylene production in living systems

Several reviews (Burg 1962, Burg, and Burg 1965, Meheriuk 1965, Thompson 1966) indicate that it is possible that all organisms produce ethylene. A large number of micro-organisms (Pratt 1944, Fergus 1954, Nickerson 1948, Biale 1960, Dimond, and Waggoner 1953, Freebairn, and Buddenhagen 1964), as well as plant, and animal tissues synthesize the gas (Chandra, and Spencer 1963a, 1963b, Gibson 1963, Kokanov 1960).

One production site of ethylene appears to be the mitochondrion (Chandra, and Spencer 1963a, 1963b, Gibson 1963). Levels of ethylene production by mitochondrial preparations have ranged greatly, with much of this variability attributed to the techniques used in mitochondrial preparation, and collection and analysis of the gas.

The crude enzyme powder prepared from bean cotyledon mitochondria (Thompson 1966) has indicated that a pathway for ethylene synthesis in bean cotyledon mitochondria involves conversion of  $\beta$ -alanine by a transaminase to malonic semi-aldehyde, which is transformed through  $\beta$ -hydroxy propionic acid, and acrylic acid to ethylene.





An energy source, apparently ATP, is required for formation of ethylene, as shown by the effect of DNP upon pre-climacteric fruit (Spencer 1959), and requirement in enzyme systems prepared from tomatoes (Meheriuk, and Spencer 1964). If sufficient ATP is added, anaerobic conditions, or DNP, which blocks the formation of ATP (Weinbach, and Garbus 1965), will not affect ethylene production (Meheriuk 1965).

Tissues in a diseased condition that resembles senescent tissue to the extent that there is a structural breakdown, produce substantial quantities of ethylene. Botrytis infection of carnations causes an increased synthesis of ethylene (Smith, Meigh, and Parker 1964), as does wounding as in the dissection of cantaloupe (Pratt, and McGlasson 1964).

As mentioned in the section on auxin-ethylene interactions, sub-lethal doses of 2,4-dichlorophenoxy-acetic acid, or of 3-indole acetic acid increases the rate of ethylene production by the cotton plant (Hall, and Morgan 1963). This may be comparable to wounding.



## Mitochondria

A large number of excellent reviews on mitochondria have recently appeared, and serve to indicate the complexity of mitochondrial metabolism (Lehninger 1964, Singer 1966, Hatefi 1966, Okunuki 1966 Green 1966, Slater 1966, Tager, Papa, Quagliariello, and Slater 1966, Chance, Estabrook, and Williamson 1965).

In higher plants and animals, the major portion of the processes responsible for the controlled oxidation of respiratory substrates, the conservation of the energy thus released, and the transfer of this energy in usable forms to the various energy requiring processes in the cell, is carried out in a membrane enclosed vesicle called a mitochondrion. Much of the tricarboxylic acid cycle, the Embden-Meyerhof-Parnas pathway, the lipid synthetic, and degradative systems, and the components of the electron transfer chain, and phosphorylation mechanism is intimately connected with this small body. Millerd,





Bonner, Axelrod, and Bandurski (1951) were among the first to prepare active particulate systems from higher plants. Much of the research on mitochondrial metabolism has been done with animal tissue, hence the mitochondrion from plant tissue is only now becoming as well understood as its close relative from animal tissue.

Mitochondria can exist in a variety of shapes and sizes, however are reasonably uniform within a given cell type (Novikoff 1961). Parsons, Bonner, and Verboon (1965) found that active mitochondria prepared from a fairly wide range of plant tissues were spherical. Earlier workers found that mitochondria ranged in shape from a "dumb-bell" form to a very elongated form (Farrant, Porter, Robertson, and Wilkins 1956, Palade 1953). Many of these shapes can be explained as results of the preparatory, and fixation techniques used at that time. Palade (1953), Mercer (1960), Novikoff (1961), and Parsons et al (1965) have confirmed that the plant cell mitochondrion has the same type of double membrane as animal cell mitochondria. Richardson, Tappel, Smith, and Houle (1962) have indicated





that animal mitochondria from a great variety of species, differ only slightly in the composition of the lipid part of the membrane. However, sweet potato mitochondrial lipids were found to be much more saturated than lipids prepared from any of the types of animal mitochondria. This may have a very definite bearing on the response of plant mitochondria to certain agents or treatments.

#### Mitochondrial development and senescence

Opik (1965) found an increase in oxygen uptake by intact bean (Phaseolus vulgaris L.) cotyledons. Oxygen uptake increased to a maximum between the third and fifth day after addition of water to the seeds. However, mitochondrial respiratory activity fell after thirty-six hours after addition of water, and electron microscopy indicated that in the storage cells, the mitochondrial cristae had swollen, and the matrix had darkened by the third day. The mitochondria of the vascular bundles did not undergo this change until the fifth day, thus explaining the decrease in respiration from the fifth day on.

Akazawa, and Beevers (1957), using Ricinus



zanzibarensis L. cotyledons, Hanson, Vatter, Fisher, and Bils (1959), using Zea mays L. scutellum, Howell (1961), using Glycine max L. cotyledons, and Young, Huang, Vanecko, Marks, and Varner (1960), using Pisum sativum L. seedlings, were able to show an increase in mitochondrial activity during germination as well. In these studies, activity has generally been measured as the amount of oxygen uptake per unit weight of mitochondrial protein, hence are open to errors inherent in the preparatory techniques. Cherry (1963) found that the increase in activity was paralleled by a visible increase in the number of cristae in mitochondrial preparations from various stages during the development of peanut cotyledons. A similar result was found with Arum maculatum L. spadix (Simon 1959, Simon, and Chapman (1961).

Spencer, and Olson (1965) have shown ethylene production to roughly parallel carbon dioxide production in germinating castor beans. As mitochondria appear to be a major centre for ethylene synthesis, such an increase fits well with the increase in activity shown by Akazawa, and Beevers (1957).

As most higher plants do not allow extensive mutational or environmental modifications in their







cellular respiratory systems, much of the research on the formation of mitochondria is based upon lower organisms. Saccharomyces cerevisiae is very useful in this respect in that large modifications in cellular mechanisms can be induced through mutations, and environmental changes. Linnane, Vitals and Nowland (1962) have shown the apparent absence of mitochondria in anaerobically grown Torolupsis utilis, another of the commonly used yeasts. However, an intra-cellular membrane system was present, which may have been the mitochondrial precursor. Schatz (1963) was able to identify such particles in preparations from aerobically grown S. cerevisiae containing various combinations of the components necessary for a complete respiratory system. He suggests that three types of particle are present. The first mitochondrial precursor contains only primary dehydrogenase, and conversion to the second type of precursor is inhibited by anaerobiosis. The second type is similar to the first, but contains the basic electron transfer system except for the succinate-cytochrome c reductase enzyme system. The third type is the fully developed mitochondrion. The



amount of each would depend on the age of the cell, and the conditions under which it is grown.

Certain small flagellates of the genus Micromonas, which contain only one mitochondrion per cell, are known to have division of this mitochondrion on division of the cell (Manton 1961). Whether or not this "direct" division occurs in higher organisms is not known, and much of the literature seems contradictory.

Schjeide, McCandless, and Munn (1964), using chicken oocytes, were able by electron microscopy to follow the formation of a mitochondrial-like structure from micro-vesicles. However, the final particulate resembled a mitochondrion only in being vesicular, with slight infoldings in the inside membrane. Yatsu (1965) has shown that mitochondria in dry cottonseeds have a normal appearance even after a year under anaerobic conditions. This may indicate that synthesis de novo is not probable in seed tissue on germination.

Luck (1965) was able to demonstrate that division is the predominant mechanism in Neurospora crassa. He used pulse labelling to show the incorporation rate of mitochondrial lipids to be very close to the rate of increase in mitochondrial number. This would





suggest an uptake of lipid material resulting in an increase in size, and then division of the mitochondrion into smaller mitochondria.

Pathological conditions in plants appear to increase the mitochondrial content (Verleur, and Uritani 1965). This would account, in part, for the increase in respiration after infection, and discount the possibility that uncoupling of oxidative phosphorylation has taken place. Wounding will cause a similar increase in mitochondrial content (Asaki, Honda, and Uritani (1966), as does ageing of potato tubers (Lee, and Chasson 1966). These results may of course reflect only differences in ease of preparation from the various types of tissue.

It would thus appear that the synthesis of mitochondria is not at all understood as yet. Most probably, mitochondria increase in number through initial development from a vesicular precursor, and subsequent divisions. The initial formation of the mitochondrion must occur during the early stages of cell development in embryonic tissue (Schjeide et al 1964).





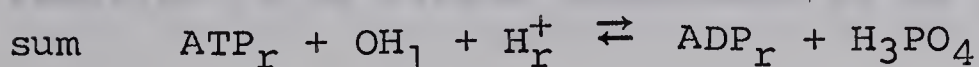
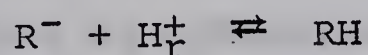
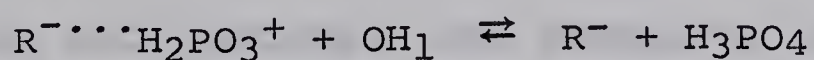
## Oxidative phosphorylation

Plant mitochondria prepared from a variety of tissues have been shown to actively oxidize malate, succinate, and NADH, with ADP control of the respiratory rate (Wiskich, and Bonner 1963, Sikes, and Bonner 1965), whereas other tricarboxylic acid cycle intermediates are oxidized slowly. Whether this is an artifact of the isolation techniques is not known. Animal mitochondria were not believed able to metabolize added NADH until recently, but Blanchaer, and Griffith (1966) found NADH to be oxidized by pigeon heart mitochondria showing some of the accepted criteria for intact mitochondria (high ADP/O ratio, and high respiratory control ratios) (Chance, and Williams 1956). ADP is able to increase the respiratory rate, hence oxygen uptake, in a system having oxidation tightly coupled to phosphorylation. This control is probably the best criterion of coupling available, and is easily determined by polarographic methods (Lehninger 1964). Similarly, the uptake of phosphate can be compared to the amount of oxygen taken up, to give a phosphate to oxygen uptake ratio (P/O), which is also a criterion of good mitochondria, and can also



be used to determine the number of phosphorylation sites in the mitochondrial electron transfer chain. The methods involved are well discussed by Bonner (1965), and will not be recounted here.

The most plausible theory of oxidative phosphorylation, that of Mitchell (1961, 1966a, 1966b), does not involve hypothetical high energy carriers, and is the cause of much research in many laboratories. The theory is based on the premise that the function of the respiratory chain is to produce a low concentration of hydrogen, and hydroxyl ions in the region of an adenosinetriphosphatase located in the mitochondrial membrane, so that the ATPase can catalyze the synthesis of ATP. This ATPase is accessible to hydrogen ions, ADP, ATP, and phosphate from one side of the membrane, and to hydroxyl ions from the other side of the membrane. Water can not enter from either side. Slater (1966) has given the reaction as follows:



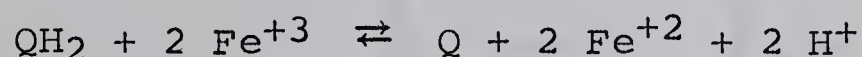
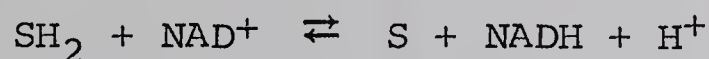






The suffixes serve to indicate a side (left or right) of origin, and  $R^-$  is a nucleophilic grouping of the ATPase. The reaction is written for undissociated ADP, ATP, and phosphate as one would expect in a nonaqueous medium. The ionic species present on the right hand side will be governed by the pH on that side. At pH = 7.5, an additional acid radical is formed on hydrolysis.

The two reactions producing hydrogen ions in a simple system are as follows:

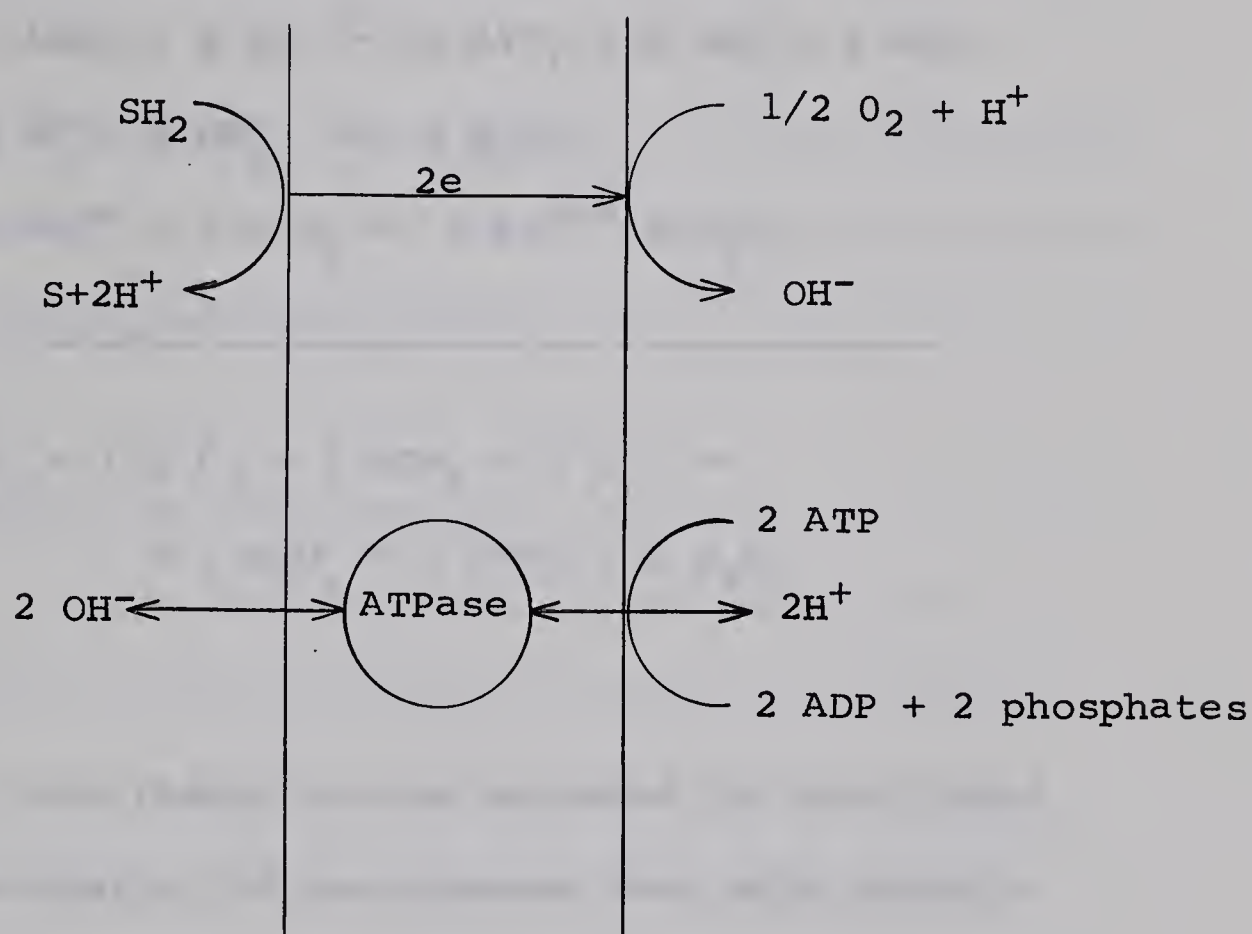


$SH_2$  refers to a reduced substrate, such as succinate,  $QH_2$  and  $Q$  refer to the reduced, and oxidized forms of ubiquinone, and  $Fe^{+2}$ , or  $Fe^{+3}$  refer to the cytochromes.

Mitchell suggests that hydroxyl ions are removed from the "left" side of the membrane by operation of the electron transfer chain, and hydrogen ions are removed from the "right" side by reaction with hydroxyl ions formed by the reduction of oxygen by cytochrome c oxidase. This latter reaction is of little importance at pH = 7.5.



For example, succinate oxidation, shown below, where succinate, and succinate dehydrogenase, and ubiquinone reduce two molecules of cytochrome b ( $\text{Fe}^{+3}$ ) on the left hand side of the membrane, which must by the above conventions, contain the respiratory chain in a specific orientation.

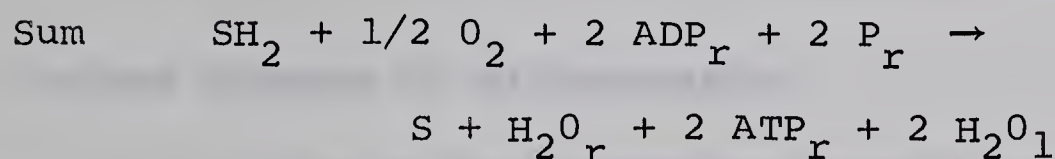
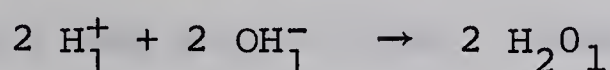
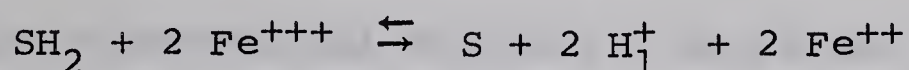


The hydrogen ions formed by the reactions given previously, are unable to diffuse through the membrane, and react with hydroxyl ions on the left hand side. The electrons travel along the cytochrome chain, and react with the production of hydroxyl ions at the other side of the membrane. The hydroxyl ions are similarly unable to pass through the membrane, and are neutralized by



hydrogen ions on the right hand side. The removal of hydroxyl ions from the left side, and hydrogen ions from the right side drives the ATPase reaction to form ATP.

The reaction can also be written as:



This theory can be extended for NAD linked substrates easily, if one assumes that NADH travels freely through the membrane.

Mitchell's theory explains respiratory control, for in the absence of ADP or phosphate, no hydroxyl ions are formed on the substrate side of the membrane to neutralize the hydrogen ions, hence a membrane potential capable of inhibiting passage of electrons along the respiratory chain would occur,





through exchange of cations for the excess hydrogen ions.

Mitchell's theory appears far less complicated than present evidence indicates oxidative phosphorylation is. Recent research has shown that the pH of an anaerobic mitochondrial suspension decreases temporarily when a small amount of oxygen is added (Mitchell, and Moyle 1965a, 1965b). Further discussion of this theory is given in the sections on mitochondrial volume changes, and ion uptake.

#### Volume changes of mitochondria

One of the metabolic control mechanisms in the cell may be the swelling and shrinking phenomena shown by mitochondria (Ernster, and Lindberg 1958). An increase in volume would result in an increase in permeability of the mitochondrial membrane, as well as an increase in ease of contact with the enzymes found in the membrane. The result would be an increase in respiration. The opposite is found when the mitochondrion is shrunken.

There are two general types of volume changes



shown by mitochondria; a passive change, which is very rapid, in response to changes in tonicity of the suspending media, and an active change, relatively quite slow, which is closely related to the respiratory rate.

The first quantitative treatment of passive, or osmotically induced volume changes of mitochondria was by Tedeschi, and Harris (1955). They found that mitochondrial volume varies inversely as the osmotic pressure of the medium, in an almost linear fashion, if one assumes that mitochondria have an osmotic dead space of forty to fifty percent of their volume. This is believed to correspond to the volume of solids making up the mitochondrion. This may be an oversimplification, because of the heterogeneity of an isolated mitochondrial preparation, and changes in volume resulted from endogenous respiration, however this method has given much information on mitochondrial permeability. Cleland (1952), and Watanabe, and Williams (1953) found that sucrose, and mannitol penetrate very slowly, whereas ethylene glycol, glycerine, and sodium, potassium, and chloride ions penetrated rapidly. Tedeschi, and Harris (1955) determined that the rates





of penetration were in approximate proportion with the olive oil - water partition coefficient of the compounds listed. Their results agree well with studies on other membranes, that is, that entry was favoured if the material was lipid soluble.

Sucrose and other osmotic agents do penetrate into the mitochondrion (Werkheiser, and Bartley 1957, Amoore, and Bartley 1958, Amoore 1958), but at a very slow rate. Malamed, and Recknagel (1959) were able to reconcile this factor in their observation that although about two-thirds of the water volume of the particulates in their preparation was slowly permeable to sucrose, the remaining space behaves as an ideal osmometer. Tedeschi (1965) has pointed out that these results are still fairly consistent with a single compartmented structure, because of the wide range of size, and intactness found in mitochondrial preparations.

When EDTA is added to the suspending medium, diffusion of sucrose across the membrane is blocked. However, if substrates for the electron transfer chain, or agents causing an increase in mitochondrial volume are added, sucrose transport is increased (Lynn, Fortney, and Brown 1964).



Active changes in volume depend entirely upon the amount of mitochondrial respiration. This is shown by the facts that anaerobiosis, and respiratory inhibitors inhibit swelling (Hunter, Davies, and Carloti 1956, Lehninger, and Ray 1957, Lehninger 1962, 1964). For instance, uncoupling of phosphorylation by low concentrations (22  $\mu\text{M}$ ) of DNP causes swelling, presumably by rapidly depleting oxidizable substrates. However, when high concentrations (100  $\mu\text{M}$ ) are added, swelling is inhibited, as is respiration. This indicates that respiration but not phosphorylation is required for active swelling (Hunter 1963). Lipsett, and Corwin (1959) found that the addition of any one of the necessary components for oxidative phosphorylation (phosphate, substrate, or phosphate acceptor) will cause swelling. If all are added, swelling is essentially equal to that when none of the above are added, hence oxidative phosphorylation protects against excessive swelling.

The mechanism by which respiration supports swelling is not known. Hunter et al (1956), and Lehninger et al (1957), to explain the swelling linked depletion of pyridine nucleotides, proposed that the reduced state of the respiratory carriers such as NAD could be the





factor involved in mitochondrial resistance to swelling. Although NADH enhances swelling (Di Sabato 1959), passage of electrons through pyridine nucleotides is not essential, for substrates such as succinate will support swelling when amytal is used to block NADH oxidation (Hunter 1963). Present work implicates the flow of electrons through the respiratory chain as being more important than the state of the carriers (Chappell, and Greville 1958, 1959, Hunter, Fink, and Hurwitz 1959, Hunter, Levy, Fink, Schulz, Guerra, and Hurwitz 1959, Corwin, and Lipsett 1959).

Cereiyo-Santalo (1966a) has suggested that protons produced by electron transfer are the critical factors in respiratory dependent swelling, and that their concentration modifies protein conformation. In a more exhaustive analysis of the effect of media pH on mitochondrial swelling this possibility was extended to the effect upon a protein gel (Cereiyo-Santalo 1966b). At acid pH, swelling in isotonic potassium chloride was completely independent of respiration. The swelling appears to be the result of the formation of a new Gibbs-Donnan equilibrium inside the mitochondrion, hence a change in structure occurs that is analagous to that undergone by a protein gel with changes in pH.





Sucrose is inhibitory to spontaneous swelling at pH = 7.4, and that induced by thyroxine (Lehninger, Ray, and Schneider 1959). They proposed that sucrose blocked an enzymatic step in the coupling of respiration with membrane permeability, and contractility.

Potassium chloride media at pH = 7.4, similarly inhibits swelling caused by endogenous respiration, but this can be overcome by addition of respiratory substrates (Cereijo-Santalo 1966a), which would activate transfer of ions across the membrane.

Dallam, and Slater (1966), and Packer, and Tappel (1960) provided evidence for the presence of a contractile protein whose energy was supplied by an ATPase. Ohnishi, and Ohnishi (1962) succeeded in isolating from rat liver mitochondria a contractile protein with properties similar to actinomysin. Aoki, Burgos, and Tellez de Inon (1965) established that this protein was found in the mitochondrial matrix, and one can conclude that this may act in a similar manner to a protein gel.

Two types of active swelling are known, and differ to some extent in the morphological and biochemical changes involved. Most of the studies have been on the



so-called "large amplitude changes", instead of the "low amplitude changes" that are believed to be physiological (Lehninger 1962).

Wlodawer, Parsons, Williams, and Wojtczak (1966) have shown that high amplitude changes in volume result in irreversible changes in mitochondrial structure. In a phosphorylating system, AMP, and/or ADP produce less large amplitude swelling than succinate alone (Lipsett, and Corwin 1959). DNP causes rapid swelling if added after AMP, but has no effect if ADP is present. If uncoupling were the cause, there should be no difference, thus an auxiliary enzyme must be present. Such an enzyme is the adenylate kinase system, where AMP could react with ATP to form two molecules of ADP, or two molecules of ADP could form one molecule each of AMP and ATP. Hence ATP can be implicated as a control factor, for while treatment with DNP would decrease ATP levels, sufficient ATP to control swelling would still be present, if ADP was added, and an adenylate kinase was present. The problem is of course whether ATP directly influences swelling, or whether it affects another system such as the contractile protein, that may be responsible for volume changes.







ADP has been said to reverse swelling in phosphorylating systems (Packer 1959, 1962). However, Longo, and Arrigoni (1964) found that ADP would not substitute for ATP in reversing swelling. Part of this problem is evident in light scattering recordings made by Packer (1963). ADP causes very rapid shrinkage, then swelling as phosphorylation slows down because of lack of substrate.

Phosphate may induce swelling at concentrations as low as one millimolar (Hunter, and Ford 1955), and this process is closely related to enzymatic uptake during oxidative phosphorylation. As arsenate, which acts as a competitor for phosphate in oxidative phosphorylation, also causes swelling (Hunter, and Ford 1955, Lehninger 1959), and is known to form an unstable intermediate which breaks causing uncoupling (Crane, and Lipmann 1953, Wadkins 1960), this relation to enzymatic uptake is confirmed. ADP, and ATP are both able to counter the swelling effect of phosphate, however, other nucleotides are not as active (Connelly, and Hallstrom 1966).

Slater (1957), and Tapley (1956) found that calcium causes a specific type of swelling, because



other similar divalent ions inhibit mitochondrial swelling. Calcium ions appear to stimulate swelling by increasing the production of an endogenous swelling factor, and not by uncoupling (Wojtczak, and Lehninger 1961).

Free fatty acids play an important role in swelling, for they cause a stimulation of ATPase activity (Pressman, and Lardy 1956), and uncoupling of oxidative phosphorylation. The effect depends greatly upon chain length, and degree of unsaturation of the fatty acid (Zborowski, and Wojtczak 1963). Lehninger, and Remmert (1959) have prepared from mitochondria a fatty acid fraction capable of causing swelling, and uncoupling. Its formation seems to depend on the amount of calcium ions in the medium (Wojtczak, and Lehninger 1961). Low concentrations of BSA have been shown to inhibit the swelling induced by calcium, or free fatty acids, however have no effect on swelling induced by phosphate, glutathione, or carbon tetrachloride (Wojtczak, and Lehninger 1961, Tapley 1956, Lehninger, Ray, and Schneider 1959). It is interesting to note that when ATP is added to spontaneously swollen mitochondria, free fatty acids are incorporated into





phospholipids. Sucrose inhibits this uptake (McFarlane, Gray, and Wheelden 1960).

GSH, GSSG, cysteine, and other disulfides cause swelling in a somewhat different manner than other swelling agents (Neubert, and Lehninger 1962, Lehninger, and Schneider 1959). Since heavy metals, iodoacetamide, and  $\rho$ -chloromercuribenzoate also cause a similiar effect (Dickens, and Salmony 1956, Tapley 1956), one can suggest that -SH and -S-S- groups are involved in swelling. Various disulfide hormones (oxytocin, and vasopressin) greatly increase swelling (Lehninger, and Neubert 1961), indicating that unhindered disulfides may act through attachment with the large numbers of free -SH groups in the various mitochondrial proteins (Riley, and Lehninger 1964). GSH has also been shown to cause loss of a heat labile factor necessary for mitochondrial contraction into the medium, where it becomes greatly diluted (Lehninger 1962, Lehninger, and Gotterer 1960).

Ascorbate acts in a similar manner to GSH (Hunter, Levy, Fink, Schulz, Guerra, and Hurwitz 1959), and studies with analogs of it allow the conclusion that its action is based on its activity as an electron





donor at the cytochrome c level (Hunter et al 1959, Lehninger, ul Hassan, and Sudduth 1954).

Long exposure of mitochondria to ascorbate (Hoffsten, Hunter, Gebicki, and Weinstein 1962), ferrous ions (McKnight, Hunter, and Oehlert 1965), and GSH results in an increase in lipid peroxidation, as well as in the rate of mitochondrial swelling. Phosphate inhibits the lipid peroxidation induced by GSH (Hunter, Weinstein, Scott, and Schneider 1963). Neubert, Wojtczak, and Lehninger (1962) have identified a factor ("C factor") required for mitochondrial contraction as a GSH peroxidase. It is thus possible that if this factor is not present, then GSH aids peroxidation. Phosphate apparently acts as an antioxidant under these conditions. Lipid peroxidation is an essentially non-reversible reaction and results in eventual membrane disruption, and mitochondrial disintegration (Schneider, Smith, and Hunter 1964). The fatty acids of mitochondrial membranes are highly unsaturated. For instance, beef heart mitochondrial fatty acids have about 1.7 double bonds per fatty acid residue, hence are quite sensitive to peroxidation (Holman, and Widmer 1959). Schneider, Smith, and Hunter (1964), and Tappel, and Zalkin (1959)



have shown that lipid peroxidation is closely associated with swelling and membrane disruption. Ageing of rat liver mitochondria caused an increase in the amount of lipid peroxidation, though increasing the concentration of mitochondria inhibited the peroxide formation (Thiele, and Huff 1960).

ATP induced contraction of mitochondria is inhibited by oligomycin A, atractylate, sucrose, and azide, hence at least a portion of the oxidative phosphorylation mechanism is essential for contraction of mitochondria (Lehninger 1962). ATP is hydrolyzed to ADP and phosphate during contraction, and as many as three hundred moles of water are forced out of the mitochondrion per mole of ATP hydrolyzed (Lehninger 1959, 1964). However, since ATPase activity is fairly high in swollen mitochondria, and continues after water extrusion is complete, one can not conclude that hydrolysis is required (Lehninger 1959).

ATP seems to affect the membrane rather than the matrix of the mitochondrion, as it is able to cause light scattering changes in small fragments of mitochondria (Packer, and Tappel 1960, Gregg and Lehninger 1963).

In general, mitochondria will shrink when







treated with magnesium ions, and manganese ions. Both inhibit swelling strongly (Tapley 1956, Rossi, and Zatti 1960). EDTA, which forms chelates with divalent ions inhibits most types of mitochondrial swelling (Tapley 1956, Gallagher 1960). In plant mitochondrial preparations, it is particularly required because of the high calcium content (Bonner 1965).

Low amplitude, oscillatory changes in volume occur in tightly coupled mitochondria (Cereijo-Santalo 1966c). These changes are completely reversible (Beechey, and Hollen 1959, Chance, and Packer 1958, Holton 1957, Packer 1960, 1961), are not inhibited by sucrose, and are somewhat more rapid than large amplitude changes (Packer 1960). Uncoupling agents cause rapid contraction (Packer 1960). Lehninger (1962) has suggested that the very small changes are reflections of structural modifications in the inner elements of the mitochondrion, and do not reflect actual size changes. Hackenbrock (1966) has shown that only interior structural changes occur as the mitochondria pass through the respiratory states I to IV, as defined by Chance, and Williams (1956). These low amplitude changes thus appear to more closely resemble "in vivo" changes in mitochondrial volume,



than do the large amplitude changes discussed earlier. Low amplitude changes also fit better with the protein gel theory advanced by Cereijo-Santalo (1966c), and evidence has been presented that a transfer of hydrogen ions out of the membrane occurs during swelling (Mustafa, Utsumi, and Packer 1966 ). Such a transfer may also serve as a further proof for Mitchell's theory (Mitchell 1966), as well indicating that the protein gel theory is a probable mechanism for low amplitude swelling at least.

There are a number of systems that are analagous to mitochondria, in that they undergo volume changes. For instance, bacterial protoplasts change in volume in response to changes in the osmotic concentration of the medium in which they are suspended. As well, they will swell in conjunction with an increase in respiration, and will shrink spontaneously when respiratory substrate is depleted (Abrams 1959,1960, Packer, and Perry 1960). This suggests that volume changes are due only to changes in the osmotic concentration inside, or outside of the protoplast. Erythrocyte "ghosts" change their shape in vitro in the presence of ATP, possibly through the action of an ATPase (Nakao, Nakao, Tatibana, and Yoshikawa





1960, Nakao, Nakao, Yamazoe, and Yoshikawa 1961).

Chloroplasts exhibit both passive volume changes in response to their osmotic environment, and active changes caused by energy dependent mechanisms (Nishida 1963, Packer, Seigenthaler, and Nobel 1965). Light scattering changes can also be induced in chloroplast membrane fragments (Gross, and Packer 1965).

#### Ion uptake

Excellent reviews of mitochondrial ion uptake are given by Lehninger (1964), and Harris, Judah, and Ahmed (1966), primarily for animal preparations.

The ability of isolated plant mitochondria to accumulate ions against a concentration gradient was shown by Robertson, Wilkinson, Hope, and Nesztel (1955). The process appears to be a respiration dependent process (Millerd, Wiskich, and Robertson 1964).

Plant mitochondria have shown only slight increases in oxygen uptake on addition of divalent ions (Bonner 1965), compared to those prepared from animal tissues (Chappell, Cohn, and Greville 1963). Valinomycin treated animal mitochondria show rapid transfer of potassium, and calcium ions (Moore, and





Pressman 1964, Ogata, and Rasmussen 1966). A similar potassium ion transfer effect is initiated in plant mitochondria by valinomycin. However, there is no response to calcium addition, and Bonner (1965) has suggested that this may be a result of the mechanism by which calcium is immobilized in plants.

Magnesium ions taken up in conjunction with phosphate appears to precipitate inside the mitochondrion as  $Mg_3(PO_4)_2$  (Millard et al 1964). The significance of this is not understood.

The theory of active transport in mitochondria depends greatly upon the elucidation of the mechanism of oxidative phosphorylation, and thus is not at all complete. In an active transport process, it is sufficient to transfer only one of an ion pair (Robertson 1960, Mitchell 1961), and one would expect the anion to follow passively. In the case of phosphate supported magnesium uptake, phosphate entry is suggested to be secondary to magnesium uptake, because of evidence of the competitive effects of other ions (Millerd et al 1964). When phosphate is not present, hydrogen ions are ejected out of the mitochondrion to maintain the charge equilibrium (Moore, and Pressman 1964). This



is in agreement with Mitchell's theory of phosphorylation for which charge separation is essential (Mitchell 1966a).

Carafoli, Gamble, and Lehninger (1965) have shown that potassium dependent oscillations in respiration linked movements of calcium and hydrogen ions in rat liver mitochondria are present, and that there may be a feedback relationship between active calcium ion uptake, and the calcium efflux rate. There was little stoichiometry between calcium uptake and electron transfer in the study by Carafoli et al (1965). However, later results by Carafoli, Gamble, Rossi, and Lehninger (1966) indicate that changes in the ionic concentration of the medium, such as pH changes, have no modifying upon the molar ratio of hydrogen ions ejected per calcium ion taken up. Under normal conditions (pH of 7.4), approximately one hydrogen ion is ejected for each calcium ion taken up, or the ejection of one pair of electrons ( $2 H^+$ ) per site, or six hydrogens for the full respiratory chain occurs with every calcium ion taken up (Chappell, and Crofts 1965, Chance 1965).

Snoswell (1966) has shown that respiring mitochondria eject hydrogen ions at a constant rate. When ADP is added to the mitochondrial suspension, this







ejection ceased, and the hydrogen ion concentration decreased during the phosphorylation of ADP. Ejection of hydrogen ions is dependent on respiration, and uncoupling agents, while increasing both respiration and hydrogen ion ejection, decrease the ratio of hydrogen transfer to electron transfer, indicating a breakdown in the hydrogen ion transfer mechanism.

Corn root mitochondria are able to actively accumulate calcium, and phosphate, with competition between their uptake, and ATP formation. Phosphate was required for calcium uptake, and accumulation of magnesium and phosphate could not be shown unless calcium was present (Hodges, and Hanson 1965).

#### Adenosine triphosphatase (ATPase)

The driving force for active ion uptake has been related to ATPase activity of various tissues (Lehninger 1964). ADP inhibits ion uptake by driving oxidative phosphorylation, apparently by competing for some part of the coupling mechanism between oxidation and phosphorylation, or by blocking a site necessary for ion uptake. Oligomycin, one of the true uncouplers, removes much of this inhibition (Brierley 1963).

Substrate supported ion accumulation as well as ATP



supported ion uptake can occur in heart mitochondria. The former is not inhibited by oligomycin, whereas the latter is. In terms of the high energy intermediate theory of oxidative phosphorylation, both reactions would form an activated group, to which ATP must be attached if ion uptake is to occur. The theory advanced by Mitchell (1966a) suggests that oligomycin impairs ATP transport into the membrane, where the enzyme system responsible for ion uptake is located.

ATPase activity in animal mitochondria is stimulated by DNP, and other uncoupling agents, and is believed to be a reactions resulting from the reversal of the system involved in formation of ATP. Hydrolytic cleavage of an intermediate in the coupling mechanism is also suggested (Lehninger 1964).

Although three ATPases corresponding to each of the coupling sites in oxidative phosphorylation, were initially thought to be present, present evidence indicates that there is probably only one (Hemker 1962). Mitchell's theory dispenses with all but one ATPase as well (Mitchell 1966a).

The effect of DNP on mitochondrial ATPase can be explained by the Mitchell theory if one assumes that





the ATPase is accessible to the undissociated phenol on the right hand side, to the phenolate ion on the left hand side, and that the membrane is freely penetrated by the undissociated phenol. Hemker (1962) has shown that the uncoupling activity of nitrophenols is correlated with solubility of the undissociated phenol in mitochondrial lipids. The undissociated phenol thus appears to replace hydrogen ions, and since the phenolate ion can only be discharged on the left hand side, drives the ATPase reaction towards breakdown of ATP (Slater 1966).

Very little work has been done on plant mitochondrial ATPase. Forti (1957) in a general analysis, found that pea internode mitochondria had an ATPase similar to that found in animal mitochondria, that is, stimulated by mitochondrial damage, or DNP. In animal mitochondria, either magnesium, or calcium ions can activate mitochondrial ATPase; however, if both are added simultaneously, calcium ions apparently compete with magnesium ions for ATP, hence an inhibition occurs. This is similar to ATPase enzymes prepared from other non-mitochondrial animal tissues. Hanson, Malhotra, and Stoner (1965) found that calcium would not





stimulate ATPase activity in corn scutellum mitochondrial preparations. However, calcium also had no effect on mitochondrial volume changes, hence it is probable that the preparation was damaged in some way. A magnesium activated ATPase has been prepared from yeast, and is stimulated by DNP (Kotel'nikova, and Zuyagel'skaya 1964).

#### "Soluble" Adenosine triphosphatase

Pullman, and Penefsky (1963) have described an enzyme that couples oxidative phosphorylation, and is capable of hydrolyzing ATP. Racker (1965) indicates that this sub-mitochondrial system is a single protein, is activated by DNP, and exhibits the rather unusual property of being cold labile. When added to a particulate fraction obtained during the early stages of the enzyme's purification, the enzyme couples oxidative phosphorylation. The particulate fraction is a vesicular, sub-mitochondrial system, which contains all of the components of the electron transfer chain, except for the coupling enzyme. It is possible that this enzyme is the ATPase envisaged by Mitchell (1966a), for it exhibits similar properties, including reactivity with water (Racker 1965). The enzyme requires magnesium ions, although other divalent ions can substitute to



some extent. Only ATP hydrolysis was stimulated by DNP. Only cytosine triphosphate of the triphosphate nucleotides tested was not hydrolyzed. ADP was very inhibitory, thus suggesting a possible mechanism for respiratory control.

The activity of the enzyme was found to be about ten times that of mitochondrial ATPase stimulated by DNP, hence a large portion of mitochondrial ATPase must be hidden.

Penefsky (1964), and Racker (1965) found that the purified enzyme divided into subunits on cooling, thus explaining the loss of activity at low temperature. The subunits would reform the enzyme if warmed. At room temperature, the enzyme thus behaves as a single protein.

The enzyme has been prepared from beef heart, and rat liver mitochondria (Pullman, Penefsky, Datta, and Racker 1960).





### Preparation Media

Recently, Honda, Hongladarom, and Laties (1966) have described a buffered medium containing ten components (sucrose, Tris-acetate, ficoll, dextran 40, BSA, glutathione, cysteine, ascorbic acid, magnesium acetate, and manganese acetate) in which mitochondria can be prepared such that they exhibit changes in shape during respiration similar to the changes found in intact cells. While this medium is somewhat more complex than those normally used, it does indicate the amount of research directed towards the preparation of active mitochondria similar to those in vivo. Ideally, the medium should not modify the structure of the intracellular organelles, and should allow preparation of all the organelles present in the cell.

With animal tissues, experience has shown that the more complex systems create more problems than they solve because of the limited knowledge of the effects of the constituents on the reactions being studied. The same is probably true with plant tissue.

### Osmotic stabilizers

While this subject is discussed in more detail in the results section, some of the basic problems with



the osmotic stabilizers used at present are outlined here. As the mitochondrion responds very quickly to changes in its osmotic environment, sufficient concentrations of an osmotic stabilizer is required in the buffered media to be used in mitochondrial preparation and assay. However, sucrose is inhibitory towards mitochondrial volume changes to some extent, and can penetrate mitochondria (Tedeschi 1965). Mannitol has similar characteristics (Bonner 1965), and has been suggested for use where the tissue contains large amounts of starch (Verleur 1965, Wiskich, and Bonner 1963). Potassium chloride may cause agglomeration of animal tissue mitochondria (Schneider 1948, Kennedy, and Lehninger 1949), penetrates the mitochondrion very rapidly, and increases the ion content of the medium to a very high level. It is particularly useful when sucrose and mannitol are found to be inhibitory.

#### Hydrogen ion buffers

Phosphate compounds are commonly used as buffers for the preparation of mitochondria, but in the systems discussed in this thesis, would act as a mitochondrial swelling agent, thus introducing





an additional variable into the system. Good, Winget, Winter, Connolly, Izawa, and Singh (1966) have recently indicated that Tris (Tris(hydroxymethyl)-aminomethane), the stand-by when phosphate can not be used, may cause an inhibition of oxidative phosphorylation. Tris also inhibits glycosidase activity, potassium ion uptake in Lactobacillus (Dahlquist 1961; McLeod, and Onafrey 1954), and can chelate divalent ions (Mahler 1961). As well, Tris has very little buffering capacity below pH 7.2 . Good et al (1966) have prepared several new buffers, such as Tricine (N-Tris(hydroxymethyl)-methylglycine). At present, a member of this laboratory, R. A. Stinson, is testing a number of these buffers.

The influence of temperature on the buffering capacity of any of the buffers induces a problem that is not often recognized. If one makes a buffered medium to a certain pH at room temperature, and uses the medium when it is ice cold, its buffering capacity, and pH will differ considerably. Thus, in this thesis, the buffered media was brought to the required pH at the temperature at which they were to be used.

Wiskich, and Bonner (1963) have suggested that buffering compounds not be used, and instead, the pH of





the homogenate be monitored throughout the grinding process, and additions of acid, or base be made to maintain a constant pH. In a procedure involving a high pressure laboratory press, this would be impossible.

Other components of the medium

Hobson, Lance, Young, and Biale (1966) have stated that bovine serum albumin was required if respiratory control was to be obtained in avocado fruit mitochondrial preparations. This fruit has a very high fat content, thus possibly a large amount of free fatty acids. If so, one can infer from the evidence of Vazquez-Colon, Ziegler, and Elliot (1966) that the uncoupling action of free fatty acids generated the requirement for bovine serum albumin (BSA). BSA will bind fatty acids (Bjornthorp, Ells, and Bradford 1964), and will reverse the effect of free fatty acids on animal mitochondria (Zborowski, and Wojtczak 1963)

Ethylenediamine tetraacetic acid (EDTA) is added to the buffered medium used in homogenization of a tissue, so as to chelate the divalent ions, particularly calcium, released during the homogenization. EDTA causes rapid shrinkage of mitochondria swollen by divalent ions, in a reaction that involves a rapid



transfer of water, and the divalent ions out of the mitochondrion (Lynn, Fortney, and Brown 1964).

Cysteine was used by Wiskich, Young, and Biale (1964) instead of BSA. Reasonably good control was obtained. Cysteine will preserve the activity of -SH enzymes (Hageman, and Waygood 1959), and considering the amount of -SH groups present on the mitochondrial membrane (Riley, and Lehninger 1964), it may act to preserve membrane structure, or enzymes bound to the outer membrane. The addition of reducing substances, such as cysteine, to plant homogenates has been shown to reduce the darkening of the homogenate caused by phenol oxidase activity (Clayton 1959, Hackett 1958). This would be of value in tissues such as apple, and potato. Cysteine can also cause a rapid swelling in rat liver mitochondria that have been previously treated with thyroxin (Neubert, and Lehninger 1962).

Magnesium ions are required for oxidative phosphorylation, apparently in a co-ordination complex formed between ADP or ATP and the phosphorylation site (Lehninger 1964). The concentrations used are quite critical, for greater than ten millimolar may cause clumping of mitochondria (Lance, Hobson, Young, and Biale 1965).







### Physical Characteristics of Ethylene

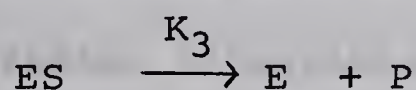
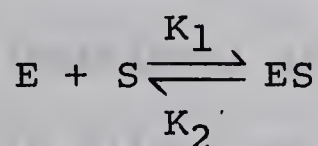
McAuliffe (1966) has determined that the solubility of ethylene in water is  $131 \pm 10$  grams per million grams of water at  $25^{\circ}\text{C}.$ , and 760 mm Hg. It has a faint, sickly-sweet odour. It is the least soluble of the common anaesthetics in water, blood, and oil. It has a blood/gas partition coefficient of about 0.14 (Eger, and Larson 1964). It is thus a useful anaesthetic for it is held in body tissues for a shorter time than the other commonly used anaesthetics (Salanitre, Rackow, Wolf, and Epstein 1965). As the water/gas, and oil/gas partition coefficients for ethylene are 0.081, and 1.28, respectively, one would expect that extended exposure to ethylene would result in a large amount of the gas dissolved in the lipids of the body.



Computer Simulation of the Effects of Ethylene  
on Mitochondrial Respiration

The behavior of biochemical systems is very difficult to analyze mathematically, unless one resorts to approximate simulation techniques, using either digital, or analog computers. Because of the size of the mechanisms studied, analog computers are normally ruled out, and thus digital computer simulation has become prominent in this field (Garfinkel 1965, Chance, Higgins, and Garfinkel 1963, Chance 1960).

Usually, simulation of an enzyme system involves setting up the differential equations corresponding to an enzyme system, or systems. For instance, the Michaelis - Menten equation can be treated as follows:



$$\frac{d(E)}{dt} = -k_1(E)(S) + k_2(ES) + k_3(ES)$$

$$\frac{d(S)}{dt} = -k_1(E)(S) + k_2(ES)$$

$$\frac{d(P)}{dt} = k_3(ES)$$





$$\frac{d(ES)}{dt} = K_1(E) \cdot (S) - K_2(ES) - K_3(ES)$$

(E) represents the concentration of enzyme E in the mechanism studied, and S, ES, and P refer to substrate, enzyme-substrate complex, and product respectively.

K' is the rate constant for a particular reaction.

When written in a form acceptable to a computer (translation languages such as Fortran, Algol, or Cobol, and assembler languages such as MAP), these differential equations can be solved within limits, using any one of a number of different types of integration procedures. The most commonly used of these numerical integration procedures is the Runge-Kutta-Gill (Gill 1951, Garfinkel, Ching, Adelman, and Clark 1966) (fourth order). A modification of this procedure, the Runge-Kutta-Merson method (Garfinkel et al 1966) (fifth order), is preferable as it allows only a very small error tolerance. A faster integration method, the point-slope method (Garfinkel et al 1966), is sometimes used, however, it is much less accurate than the first two methods given.

A major problem exists when one represents a typical enzyme with realistic physical parameters, for





the derivative becomes very small, if the error present in the calculations is to remain within a reasonable limit. One solution is to normalize the parameters by increasing the amount of work done per molecule of enzyme. If such steps are not taken, oscillations resulting from rounding off errors, and from the integration procedure itself may occur in the calculations performed by the computer (Garfinkel et al 1966). A number of programs have been written to cancel out such oscillations, without greatly modifying the physical parameters. These programs are quite slow, and give erroneous results when systems containing a normally oscillating series of reactions are simulated (Garfinkel et al 1966). Usually, the period of oscillation in the natural system (Ghosh, and Chance 1964, Chance, Hess, and Betz 1964, Chance, Ghosh, Higgins, and Maitra 1964) is much greater than that resulting from calculation.

Graphical representation of the computations is preferred, and can be done with special hardware, or programs. The latter is less time consuming, but does not give as accurate a graph (Garfinkel 1962).

Specialized programs have been written to accept modified chemical equations directly, and convert them



to the appropriate differential equations (Garfinkel, Rutledge, and Higgins 1961). These are however quite time consuming.

The Johnson Research Foundation, University of Pennsylvania, Philadelphia, has led in computer simulation of biochemical systems having begun their studies prior to 1950 (Garfinkel 1965). A number of other centres have started similar projects, but have been slowed by the cost of computer time (for an IBM 7040 - 1620 system, computer time is about \$900.00 per hour). Study of the metabolic control characteristics of ascites tumour cells (Chance, Garfinkel, Higgins, and Hess 1960), of the detailed mechanism of phosphofructokinase (Garfinkel 1966), and compartmentation of cellular components (Garfinkel 1963), are among some of the research that has been done. It is very evident from this work that the next step in enzyme simulation will involve whole cell simulation studies.





## MATERIALS AND METHODS

All chemicals used throughout the investigation were reagent grade, and were obtained from either Fisher Scientific Co., ltd., Eastman Organic Chemicals Department of Eastman Kodak Co., or Laboratory Chemicals Division of British Drug Houses, ltd. ADP and cellulase were purchased from Calbiochem, ATP, BSA, glutamic acid, glucose oxidase, and catalase from Sigma Chemical Company, and pyruvate kinase, lactate dehydrogenase, and protamine sulfate from Mann Research Laboratories, Inc. Ethylene (USP 99 %) was obtained from Ohio Chemical and Manufacturing Co. Antifoam 10 was obtained through Dr. H. Jackson from the General Electric Silicones Department, Waterford, New York.



## Choice of Tissue

### Plant tissue

Considering the latitude of Edmonton, it was felt that a tissue which could be obtained locally, at various reproduceable stages of development, would be best. Previous projects in this laboratory utilized tomatoes, but their high acidity presents problems in the preparation of subcellular particles (Meheriuk 1965)

As a follow-up to a study on ethylene production, and lipid mobilization in castor beans (Spencer, and Olson 1965), a yellow snap bean, Kinghorn wax, was tested for ethylene production. It was found that ethylene production was maximal in the germinating seed, and seedling stages at two, nine, and fifteen days (Thompson 1966). As in the castor bean study (Spencer, and Olson 1965), this correlated very well with cotyledon senescence. As well, studies with enzyme powder preparations from bean cotyledon mitochondria indicated that a mitochondrial fraction could be obtained fairly easily (Thompson 1966).

Other tissue sources, such as the potato, are readily available in the immediate area, however present problems in procuring various stages of maturity.





## Animal tissue

Particulate preparations from rat liver are among the most standard preparations used in biochemistry. Thus, with a view of testing the effects of ethylene on an "animal" system, and procuring a preparation that was relatively standard, a number of experiments were done with rat liver.

## Micro-organisms

Several types of fungi were tested. A strain of Cyathus stercoreus was found to be unsuitable for mitochondrial preparations as its cell wall was very difficult to rupture. Yeast (Saccharomyces cerevisiae) from a local brewery were then tested. The yeast preparations discussed in this thesis were made with a pure strain of Saccharomyces cerevisiae (ATCC 4111).

## Preparation of mitochondria

### Preparation of bean cotyledon mitochondria

Kinghorn wax bean seed was obtained through Steele-Briggs Seed Co., Ltd., Edmonton, and Asgrow Seed Co., Ltd., Brooks, Alberta. The seed was not treated with any chemical to preclude possible inhibitory effects in the preparations made from them.



The seeds were germinated in wooden flats filled with horticultural grade vermiculite over a layer of peat moss. Some care in planting, and packing the flat was found essential if reasonably even germination was to occur. The growth chamber was kept dark, and at a temperature of  $19^{\circ}$  to  $22^{\circ}$  C. Because of the number of flats placed in the room, the humidity was very high. Where germinated seeds one day old were required, a quantity of seed was placed under flowing tap water ( $20^{\circ}$ C.). Seeds that had not swollen after twenty-four hours of this treatment were discarded.

The age of the germinated seed was calculated from the number of hours from initial addition of water to the seeds.

The cotyledons were snapped free from the seedlings by hand. To achieve greater uniformity, cotyledons were taken only from seedlings of the same height. This method allowed some stem material to remain with the cotyledon, but this was not found to be critical. After harvesting, the tissue was washed with distilled water, and placed at  $0^{\circ}$  C.

Several problems are inherent in preparation of mitochondria from plant tissue. The cellulose-pectin





cell wall makes it difficult to rupture cells without damaging the interior components. Closely related to this is the usually acid contents of the vacuole, which on cellular rupture is often sufficient to cause irreversible damage to the mitochondria. Plants containing chlorophyll are an especially difficult problem, for chlorophyll is often freed in such quantities as to make some enzymic assays impossible.

The first of these problems can only be solved by judicious analysis of various techniques for cell rupture. Buffering of the medium used in homogenization can overcome the problem of pH changes, and the use of etiolated tissue, when possible, can remedy chlorophyll carryover into the various cell fractions.

A number of different homogenization procedures were tried, and details are given in the section on results. The final procedure adopted was the following. Two hundred grams of bean cotyledons were wrapped in four to five layers of cheesecloth, and placed in a stainless steel receptacle, along with four hundred millilitres of the grind buffer. A ram constructed of stainless steel was placed on top of the cheesecloth package, and a force of fifteen to twenty thousand pounds per square inch exerted on it by a hydraulic



press. The resulting homogenate was poured through four layers of cheesecloth into a flask. All equipment and solutions were kept at 0° C.

The homogenate was immediately centrifuged at 5000 x g in a refrigerated centrifuge (International Equipment Company, Model B-20) for ten minutes. The supernatant was poured off into another set of cold centrifuge tubes, and recentrifuged at 10,000 x g for ten minutes. The supernatant of the second centrifugation was discarded, and the pellet was gently suspended in one hundred millilitres of the suspending or wash buffer, using a large bore pipette. This suspension was then centrifuged at 2500 x g for ten minutes to remove inactive material, and starch that had not been removed in the previous centrifugations (Lyons, and Pratt 1964). The supernatant layer was then centrifuged at 10,000 x g for ten minutes to remove a particulate fraction from suspension. The pellet produced was suspended carefully in two to three millilitres of the suspending buffer by pipette, and stored in a test tube in an ice bath.

The grind medium contained 0.50 M mannitol, 0.05 M Tris-HCl, 0.001 M EDTA, 0.004 M Cysteine, and





0.1 % BSA per litre, at pH 7.6 at 0° C. The wash or suspending buffer contained 0.50 M mannitol, 0.05 M Tris-HCl, 0.001 M EDTA, and 0.1 % BSA per litre at pH 7.4 at 0° C. The assay medium used in polarographic analysis, and swelling studies contained 0.50 M mannitol, 0.01 M Tris-HCl, 0.001 M magnesium chloride per litre, at pH 7.4 at 25° C. 0.5 N HCl was used to adjust the pH of the buffered medium.

#### Preparation of potato tuber mitochondria

Potato tubers were obtained from local grocery stores, and cooled immediately prior to use. They were then peeled, and cut into slices. Essentially the same procedure as used for bean cotyledon preparations. The buffered media as developed for use with bean cotyledons was used.

#### Preparation of rat liver mitochondria

Female rats of the Sprague-Dawley strain, weighing one hundred and fifty to two hundred grams, were obtained from the Department of Biochemistry.

The rats were decapitated, and allowed to drain. The livers were immediately removed, and washed with ice-cold grind buffer. After weighing, the



the livers were cut into small pieces with scissors, and homogenized using a loose fitting Potter-Elvehjem homogenizer, using about five millilitres of grind buffer per gram of tissue. After homogenizing for one minute, and then diluting to about one hundred millilitres with grind buffer, the homogenate was centrifuged at 1000 x g for ten minutes. The supernatant was removed, and centrifuged at 10,000 x g for ten minutes. The resulting pellets were suspended in fifty millilitres of suspending buffer by pipette, and centrifuged at 1000 x g for ten minutes. The supernatant from this centrifugation was then centrifuged at 10,000 x g for ten minutes to yield a pellet that was suspended in two or three millilitres of suspending medium, and stored in a test tube in an ice bath until use. The period of this storage never exceeded ten minutes.

The buffered media developed for use in the preparation of bean cotyledon mitochondria were used.

#### Preparation of mitochondria from yeast

Yeast (Saccharomyces cerevisiae ATCC 4111) was grown in a six litre batch fermentor (Bio-Kulture Assembly, Fermentation Design Inc., Durham, Pennsylvania) with continuous stirring, and aeration at the rate of





three litres of air per minute. The growth medium consisted of 10.0 grams glucose, 5.0 grams potassium dihydrogen phosphate, 5.0 grams Difco yeast extract, and 5.0 grams Difco nutrient broth per litre, at pH 4.9 to 5.5 at 25° C. The fermentation chamber, and medium were sterilized by autoclave for twenty minutes at twenty pounds per square inch steam, and 100° C. One millilitre of Antifoam 10 (General Electric Silicones Department, Waterford, New York) was added per day to reduce foam formation in the culture vessel.

Approximately fifteen hundred millilitres of the yeast culture were drained from the culture vessel, and cooled in a cold room, while being stirred. When the temperature of the culture reached 5° C., usually about an hour after draining from the vessel, the suspension was centrifuged at 1000 x g for ten minutes. Gentle shaking, and stirring was used to resuspend the cells in five hundred millilitres of the grind buffer. This suspension was then centrifuged at 1000 x g for ten minutes. After suspension, the cells were then broken by either of the following procedures.

The yeast cell suspension was sonicated for four minutes in a Raytheon DF-101 10 KC sonic oscillator



(Raytheon Manufacturing Co., Waltham, Massachusetts), at 1.1 amps at 4° C. The resulting suspension was removed from the sonication chamber by pipette, and made to one hundred millilitres with grind buffer.

In the second procedure, the yeast cell suspension was warmed to 30° C., while being stirred. Sufficient cellulase (Calbiochem, Los Angeles, California) was added to the suspension to give a final concentration of cellulase of 0.5 %. After one and one-half hours incubation, the suspension was cooled in an ice bath, and sonicated for thirty seconds as in the first procedure. The resulting suspension was transferred to centrifuge tubes, and diluted to about one hundred millilitres with grind buffer.

The third procedure utilized a Nossal Cell Disintegrator (McDonald Engineering Co., Bay Village, Ohio). Eighteen millilitres of the yeast cell suspension were added to a Nossal capsule, along with two grams of glass beads. The capsule, and the glass beads which had been washed with de-ionized water, rinsed with 95 % ethanol, and dried at 110° C., were cooled to 0° C. prior to use. The glass beads were added slowly, with gentle stirring to allow trapped





air to escape. The capsule was filled completely to prevent foam denaturation, and placed very securely in the unit. The capsule was shaken four times, for fifteen seconds, with cooling, and a fifteen second rest period. Cooling was accomplished with a jet of liquid carbon dioxide directed towards the capsule when shaking. The suspension was poured out, and when the entire volume (about fifty millilitres) had been processed, was made to one hundred millilitres with grind medium.

The suspension prepared by any of these methods was then centrifuged, and handled in the same manner as the bean cotyledon homogenate. As with the other mitochondrial preparations, ice-cold media, and equipment was used throughout the preparation procedure, and assays were begun as soon as possible after preparation was complete.

#### Examination of mitochondrial integrity

For light microscopy, samples of the final pellet were suspended in the suspending medium, and viewed with a Reichert model Zetopan phase contrast microscope, with a 35 mm. Leitz camera attachment.

In early experiments, part of the suspension



was incubated with a final concentration of 0.001 % of Janus Green B (Allied Chemical Corporation, National Aniline Division) for twenty minutes at 25° C., and examined for colour change with the light microscope.

A procedure for electron microscopic analysis similar to that used by Parsons, Bonner, and Verleur (1965), was followed. A drop of the final mitochondrial suspension was placed on a sheet of dental wax, and a formvar, or carbon coated grid dipped through it. The grid was drained by touching it gently to a piece of filter paper. The material on the grid was fixed by placing the grid in a drop of 1.5 % osmic tetroxide for five minutes. The grid was then drained, allowed to dry in air for ten to fifteen minutes, and was then washed by passing it through a drop of demineralized water, and draining, three times. The grid was then floated on a drop of 1.5 % buffered phosphotungstic acid for twenty minutes, and then drained, and washed as before. The grids were allowed to dry, and were stored in a plastic grid box at ambient conditions. All solutions were filtered through 0.22  $\mu$  millipore filters prior to use.

150 mesh grids were used. The electron micrographs were taken with a Philips model 100 B





electron microscope. An accelerating voltage of 60 KV was used. Photographs were taken with Kodak fine grain positive film (Kodak emulsion P4-26-1) and were developed in Kodak D-19 developer.

### Measurement of Oxygen Uptake

For preliminary experiments, a commercial polarographic instrument (Sargent model III Manual Polarograph) was modified for recording by replacing the galvanometer circuit with a Sargent model SR recorder. An electrode unit was constructed for use inside the Perkin-Elmer model 202 recording spectrophotometer, and consisted of an AC-DC chopper from a car radio modified as follows. By reversing the input to the chopper, six volts (AC) as supplied by a door bell transformer, was sufficient to cause the chopper arm to vibrate at sixty cycles per second. Since the vibrating arm was isolated from the AC circuit, it was convenient to solder a platinum wire (22 gauge) to it, and complete the circuit to the polarograph by means of a shielded cable. The other half of the circuit was supplied by a calomel electrode with a long potassium chloride bridge. The electrodes were immersed in the spectrophotometer cuvette to a depth



that would not interfere with the spectrophotometer operation. The platinum electrode was coated with a commercial epoxy resin (Devcon Canada ltd., Scarborough, Ontario) after attachment to the chopper. The tip of this electrode was carefully ground flat, and was polished until smooth. The calomel electrode used was a Coleman pH meter calomel reference electrode.

The polarographic unit was set such that a voltage of 0.68 volts was placed across the electrodes, with the platinum electrode made positive, and such that full scale on the recorder corresponded to the range of 0 to 100 % saturation of the buffer with air. Since temperature strongly affects oxygen solubility, the cuvette was thermostatted at 25° C., using a holder constructed from an aluminum alloy, through which water at 25° C. was circulated (Hagihara 1961, Davies, and Brink 1942, Chance 1954, Chance, and Williams 1955a, 1955b)

In most of the experiments reported, a Gilson Oxygraph model KM (Gilson Medical Electronics, Middleton, Wisconsin) was used with the standard cuvette, and thermostat supplied with the instrument. This instrument was modified to allow use of the electrode system mounted in the spectrophotometer.





## Standardization

The amount of oxygen in distilled water is easily obtained from standard tables (Hodgman, Weast, and Selby 1966). Since the presence of chloride ions is known to influence the solubility of oxygen in water, a chemical method was tried. However, the Alsterburg modification of the Winkler method (Alsterburg 1925) was not found sufficiently sensitive, or reproduceable to use. Thus, all oxygen uptake calculations are based on the assumption usually made by persons using an oxygen electrode system, that a litre of air saturated buffered medium contains 250  $\mu\text{M}$  of oxygen at 760 mm., and 25° C.

A standardization procedure suggested by Gilson Medical Electronics was used to test operation of the machine. The test consisted of the addition of a buffer containing 10  $\mu\text{Moles}$  glucose per millilitre to the cuvette. A few microlitres of a 0.1 % solution of catalase were added after the machine had become stable. A minute later, several microlitres of a 0.1 % solution of glucose oxidase were added. The conversion of glucose would rapidly remove all oxygen from solution, and cause an appropriate change in the recording, if the machine was operating properly.



Just prior to the addition of reagents, and during mixing of the material in the cuvette, the recorder was turned off to avoid recording of mixing artifacts. During this period, the pen traced horizontal lines. Although some of the traces show discontinuities resulting from traces of oxygen stirred in during mixing, or occasionally caused by electrical disturbances, the interpretation of the results was not altered by the irregularities in the trace.

Respiratory control ratios were calculated from the amount of oxygen used when ADP is present (State III), compared to the amount of oxygen used when ADP is exhausted (State IV) (Wiskich, and Bonner 1963, Chance, and Williams 1955c, 1956, Bonner, and Voss 1961).

ADP/O ratios were calculated from the amount of ADP used compared to the amount of oxygen taken up in the same period of time (Chance, and Williams 1955a, 1955b, 1956).





## Measurement of Mitochondrial Volume Changes

A number of methods have been developed to measure changes in the size of mitochondria.

### Turbidimetric measurement of volume change

Changes in the amount of absorption of light in the visible region by suspensions of mitochondria is a simple and fairly sensitive method for measurement of mitochondrial volume changes. Early workers in the field made little attempt to calibrate this procedure with the actual volume changes. However, Tedeschi, and Harris (1955, 1958) were able to demonstrate by means of phase contrast microscopy that light absorption by a rat liver mitochondrial suspension is a function of mitochondrial volume, and the refractive index difference between the suspending medium and the particles. Mitochondrial rupture, which could cause severe deviations in measurement, was believed to be negligible during simple osmotic swelling and shrinkage. They were also able to show that if one allowed for an osmotically dead space of about forty percent of the mitochondrial volume, that the changes in mitochondrial volume obeyed osmotic law rather exactly.



Mitochondria prepared from other animal tissues do not respond as well as those from rat liver (Honda, and Muenster 1960, Chappell, and Perry 1954). However, turbidimetric changes fairly well represent volume changes in the system. Most researchers use 520 mμ as the incident wavelength, as there is little complication from respiratory components at this wavelength (Lehninger 1956, 1959).

Koch (1961) found that with osmotic swelling of bacterial spheroplasts, or mitochondria, absorbance varied nearly inversely with the volume. For objects the size of mitochondria (0.5 to 4.0 μ in diameter), little error was introduced into the calculation of volume by turbidity measurements, although the intensity of light scattered in a particular direction may be in error.

Angular light scattering measurement of volume change.

Gotterer, Thompson, and Lehninger (1961) have done a detailed study on the scattering envelope of mitochondria, and have shown that the scatter of light from mitochondria at different angles from the incident beam gives a far more sensitive method than simple turbidimetric measurements. The procedure can





reveal changes in volume not measurable with turbidity measurements, and has made possible detection of substances leaking from mitochondria that are able to cause mitochondrial volume changes. Packer (1960) has used this method to great advantage in his studies on low amplitude changes in volume. This procedure requires a very well trained operator, for calibration of the instrument, and positioning, and size of the sample can introduce large errors.

Koch (1961) was able to derive quantitative expressions for the amount of scattering, and found that for ideal spheres, approximately the size of mitochondria, that absorbance varied inversely as  $\frac{2}{3}$  (volume), approximately the relationship found by Tedeschi, and Harris (1955).

#### Packed Mitochondria volume measurements

Malamed, and Recknagel (1958), and Honda, and Meunster (1960) used centrifugation from the suspending medium in a graduated tube, similar to that used in the hematocrit method, to measure volume changes. This procedure is slow, and the centrifugation force may cause structural changes in the mitochondria.



## Gravimetric analysis of volume changes

This procedure involves centrifugation from the suspending medium, draining, and weighing of the mitochondrial pellet. Since the pellet contains some extra-mitochondrial water, a correction must be made. Werkheiser, and Bartley (1957) used a  $C^{14}$  labelled carboxypoly-glucose, which is believed not to enter into, or be absorbed upon the mitochondrion, to determine that twenty to sixty percent of the pellet may be extra-mitochondrial water. This procedure is very slow, and is not suited to continuous measurements, but it has been used to confirm absorbance measurements, and absolute water uptake (Lehninger 1959). As in the packed volume method, centrifugation may induce errors.

## Electron microscopic measurement of volume

Hackenbrock (1966), using a quick sampling procedure, and rapid embedding, was able to show definite differences in mitochondrial structure in states I, II, III, and IV, as defined by Chance, and Williams (1955a, 1956). However, the embedding and fixing of biological preparations is fraught with error. The procedure is also discontinuous, and takes an excessive amount of time to complete.





## Electronic sizing of mitochondria

Gebicki, and Hunter (1964) utilized a Coulter Counter (model B) to follow changes in the size of mitochondria with time. The counter can determine the number, and size of particles present in the system by analyzing the amount of resistance across a tiny pore ( $30\ \mu$ ) immersed in an electrolyte. Particles have a greater resistance than the medium, and as they are drawn through the pore, cause a resistance change, the intensity of which is related to size (Kubitschek 1960). The procedure is slow, and discontinuous. The very small pore required for measurement of mitochondria is easily plugged by debris, or agglomerated particles, hence the procedure is best suited as an occasional check on size.

Two of the methods discussed in the previous sections were used in this project.

## Turbidimetric analysis of volume

A model 202 Perkin-Elmer recording spectrophotometer was used to measure changes in apparent absorbance resulting from volume changes. The cuvette holder was thermostatted at  $25^{\circ}\text{C}$ . A minimal slit width



was used to reduce possible inaccuracies resulting from excessive light scattering. During the latter part of the project, a scale expansion device became available, and when coupled to an auxiliary recorder, greatly improved the readability of results. One centimeter quartz cuvettes were used throughout the experiments.

Additions of mitochondria, and substrates into the buffered medium being used were made with Hamilton microlitre syringes through hypodermic needles inserted in 22 gauge polyethylene tubing, which was passed through the case of the spectrophotometer in such a manner as not to require opening of the spectrophotometer compartment to make additions.

Where ethylene treatments were used, a portion of the test solution was saturated with the gas, and suitable dilutions were made at 25° C., using the test solution as diluent. The diluted solution was added to the cuvette with a pipette.

All measurements were made at 520 mμ.

Where changes induced by ethylene were found to be very small, the spectrophotometer was operated in a differential recording mode. Equal aliquots of the same mitochondrial suspension were added





simultaneously to each cuvette, which contained equal amounts of the suspending medium, after recording had been commenced. Additions of ethylene saturated buffer were made previously to the addition of the mitochondrial samples.

#### Electronic sizing of mitochondria

A Coulter Counter model B (Coulter Electronics Company, Florida) with a 30  $\mu$  pore was used in the tests made. All solutions used with the instrument were filtered four to five times through 0.22  $\mu$  millipore filters, and stored in sealed clean containers. This was found necessary to reduce extraneous noise resulting from dust particles in solution.

The unit was calibrated with polystyrene latex particles (Colabtex, Consolidated Laboratories (Canada) ltd.) suspended in the filtered medium used. These spheres had a mean size of 0.81  $\mu$ .

Samples to be analyzed were diluted with suspending medium, and gently added to the sample cuvette, which was thermostatted at 25° C. After mixing, the sample was counted at increments of two units over the entire range of the instrument (100 units).



### Measurement of Inorganic Phosphate

A number of different phosphate assay methods were tried. The following procedure was used initially, and is based on the method developed by Fiske, and Subbarow (1925).

A three millilitre sample was de-proteinized by the addition of one millilitre of 50 % trichloroacetic acid. This suspension was then centrifuged in a clinical centrifuge (International Equipment Company, model HN) at full speed (approximately 1000 x g) for ten minutes. A two millilitre aliquot of the supernatant was carefully removed, and one millilitre of molybdate solution, and two-tenths of a millilitre of reducing solution was added. This solution was then mixed thoroughly using a vortex mixer. After standing for five minutes, the solutions were read against a blank of distilled water treated in the same manner as the sample, at 690 mμ, with a Beckman model DU-2 spectrophotometer. One centimeter quartz cuvettes were used.

The molybdate reagent consisted of 25 grams of ammonium molybdate dissolved in 200 millilitres of distilled water, and added to 300 millilitres of 10 N sulfuric acid, and made to one litre.





The reducing agent consisted of 0.5 grams of amino-naphthol-sulfonic acid, 195 millilitres of 15 % sodium bisulfite, and 5 millilitres of sodium sulfite. This was shaken until dissolved, and stored in brown glass bottles.

In later tests, a method developed by Mozersky, Pettanti, and Kolman (1966) was used. It allowed the use of much lower phosphate levels, and gave less variable results.

The sample was pipetted into a 15 x 100 mm. test tube, and made to three millilitres with water. Three millilitres of protein precipitant was added, and the solutions were mixed, and then centrifuged at 1000 x g for thirty minutes. An aliquot of four millilitres was then taken. If less was taken, the volume was adjusted to four millilitres with diluent. One millilitre of acid perchlorate reagent was added, and the solution was again mixed. Five millilitres of IBB and one millilitre of molybdate reagent were then added, and after a thorough mixing, the solution was centrifuged at 1000 x g for thirty minutes. A Pasteur pipette was used to remove the organic phase, and place it in a one centimeter quartz cuvette.



The absorbance of the solution was read at 313 m $\mu$  against a blank of distilled water treated in the same manner as the initial sample.

The protein precipitant consisted of 1.2 M sodium perchlorate, 0.080 M glycine, and 0.2 M hydrochloric acid per litre. The diluent was made up of 0.6 M sodium perchlorate per litre. The acid perchlorate reagent contained 1.2 M sodium perchlorate, and 4.2 N sulfuric acid. IBB consisted of one volume of isobutanol, and one volume of benzene. The molybdate reagent contained 25 mM ammonium molybdate.

It should be noted that poly-hydroxy alcohols such as mannitol may complex with molybdate. An attempt was made to maintain the concentration of mannitol constant throughout the analyses as suggested by Vreman, and Jobsis (1966).

#### Measurement of Mitochondrial Adenosine triphosphatase

Mitochondria were added as a suspension in 0.5 millilitres of 0.50 M mannitol, 0.01 M Tris-HCl, and 0.001 M magnesium chloride, pH 7.4 at 25° C., to 14.5 millilitres of the same buffered medium containing 0.001 M ATP, in a 25 millilitre erlenmeyer flask. One





millilitre of this suspension (200 to 400 micrograms of mitochondrial protein per millilitre) was quickly removed, and added to the protein precipitant as used in the second method for analysis of phosphate, plus two millilitres of water. The erlenmeyer flask had previously been placed in a shaking water bath at 25° C., to allow equilibration of the reaction components. One millilitre samples were taken at intervals from this flask, and treated as the initial sample. After the incubation had been completed, these de-proteinized samples were analyzed for phosphate content. Additions of ethylene, DNP, and ouabain were made to the flasks prior to the addition of the mitochondrial suspension.

The possible hydrolysis of ATP present in the sample on addition to the protein precipitant was tested for by placing samples of the reaction mixture in protein precipitant for varying periods of time, and then analyzing for the liberation of phosphate.

#### Preparation and purification of a soluble ATPase

Mitochondria prepared as described previously from bean cotyledons, or rat liver, were used as the



source of the enzyme. One day old beans were used.

A procedure developed by Penefsky, Pullman, Datta, and Racker (1960), and Pullman, Penefsky, Datta, and Racker (1960) was followed.

Approximately three grams (wet weight) of mitochondria were suspended in fifty millilitres of 0.50 M mannitol, 0.05 M Tris-HCl, 0.001 M EDTA, pH 7.4 at 0° C. by pipette. Eighteen millilitres of this ice-cold suspension were pipetted into a chilled Nossal tube. Two grams of chilled glass beads, washed as given in the section on preparation of yeast mitochondria, were added very slowly, with gentle tapping of the tube to remove trapped air bubbles. A thin layer of Dow Corning silicone vacuum grease was applied to the inside lip of the cap of the Nossal tube. The tube, with its cap resting slightly askew were placed upright in a large test tube. Gradual evacuation of the tube was used to remove more of the air trapped in solution. When evacuation was reasonably complete, a probe fitted in the test tube's stopper was used to work the cap onto the Nossal tube.

The evacuated tube was then removed from the test tube, and placed securely in the Nossal cell





disintegrator. Ten shaking periods of ten seconds, each followed by a ten second "rest" period, were done. In every third shaking period, the carbon dioxide cooling jet was turned on for five seconds. Longer periods of cooling were found to freeze the tube's contents. After the mitochondrial suspension had all been processed (approximately thirty minutes), it was centrifuged at 20,000 x g for twenty minutes (International Equipment Company, Model B-20 refrigerated centrifuge, held at 1° C.). The pellet was discarded, and the supernatant was centrifuged at 105,000 x g for thirty minutes, at 4° C. (Beckman Instrument Co., Rotor # 40, Model L-2 centrifuge). The pellet, or particulate fraction, was suspended by pipette in fifteen millilitres of 0.50 M mannitol, 0.05 M Tris-HCl, and 0.001 M EDTA, pH 7.4 at 0° C. This suspension, and the supernatant from the same centrifugation were then centrifuged at 105,000 x g for thirty minutes at 4° C. The pellets produced were combined, and resuspended in fifteen millilitres of the same medium as before. If the supernatant poured off before suspension of the pellets was not clear, it was again centrifuged.

Samples for analysis of the protein content were taken at each step.



The clear supernatant held at 0° C. was adjusted to pH 5.4 by the slow addition of ice-cold 1.0 N acetic acid. This solution was then centrifuged at 18,000 x g for fifteen minutes, at 4° C., and the precipitate was discarded. The supernatant was adjusted to pH 6.7 with ice-cold 2 M Tris (pH 10.9).

Prior to the mitochondrial preparations, one gram of protamine sulfate (Mann Research Laboratories, Inc.) was dissolved in fifty millilitres of distilled water. This solution was adjusted to pH 7.0 with 1.0 N potassium hydroxide, and diluted to 100 millilitres with water, then cooled to 4° C. After centrifugation at 10,000 x g for ten minutes, an equal volume of water was added to the clear supernatant.

For each ten milligrams of protein recovered in the supernatant from the isoelectric precipitation step, 0.2 millilitres of the protamine sulfate solution were slowly added, with gentle stirring with a magnetic stirrer. After the addition was complete, stirring was continued for fifteen minutes at 0° C., and then the solution was centrifuged at 10,000 x g for fifteen minutes. After pouring off from the pellet, the supernatant was diluted by the same volume of the protamine sulfate solution as used earlier, and was





treated in the same manner as the first addition. With preparations from bean cotyledon mitochondria, a third precipitation was done.

The precipitates were dissolved in 0.40 M ammonium sulfate, 0.50 M mannitol, 0.01 M Tris-HCl, and 0.001 M EDTA, pH 7.4 at 25° C. to give a final protein concentration of four milligrams per millilitre. This step was carried out at room temperature. A small amount of insoluble material was centrifuged off at 1000 x g for two minutes at 25° C. To the clear supernatant, an equal volume of saturated ammonium sulfate, pH 5.5 at 25° C., was added with gentle stirring. The suspension was then placed in an ice bath for fifteen minutes to facilitate precipitation of the enzyme. The suspension was centrifuged at 10,000 x g for ten minutes at 0° C., and the pellet was carefully suspended as before. The precipitation was again carried out.

The pellet produced was suspended in 0.50 M mannitol, 0.01 M Tris-HCl, and 0.001 M EDTA to give a final protein concentration of fifteen milligrams per millilitre. After allowing the solution to warm to room temperature, 0.02 millilitre of 0.2 M ATP, pH 7.4 at 25° C. was added, and the solution was placed in a



sixty-five degree water bath for two minutes. After cooling to about 25° C. in a water bath, the suspension was centrifuged at 10,000 x g for ten minutes at room temperature. Ammonium sulfate precipitation as given before was then carried out on the supernatant to remove the added ATP.

According to Pullman et al (1960), the enzyme prepared from beef heart mitochondria was stable at 4° C. as a suspension in 50 % ammonium sulfate for a period of three weeks. The enzymes prepared in this project were thus stored in this manner.

For assays, an aliquot of the suspension was centrifuged at 10,000 x g for ten minutes to recover the enzyme, which was then suspended in 0.50 M mannitol, 0.01 M Tris-HCl, 0.001 M magnesium chloride, pH 7.4 at 25° C., and stored at room temperature.

The enzyme was assayed by a fluorometric procedure suggested by Pullman et al (1960). A Turner Model III Fluorometer (G. K. Turner Associates, Palo Alto, California) with an excitation filter (110-811) passing light below 370 mμ, and a fluorescence measuring filter (110-828) passing light above 450 mμ, was used.





0.2 millilitre of a solution containing 32 micrograms of pyruvate kinase, and 13 micrograms of lactic dehydrogenase per 0.2 millilitre, in 0.50 M mannitol, 0.01 M Tris-HCl, and 0.001 M magnesium chloride, pH 7.4 at 25° C., 0.20 millilitre of the enzyme mixture (1.0 millilitre of a suitably diluted particulate fraction, plus 0.1 millilitre of the coupling enzyme (0.100 milligrams per millilitre), and 3.0 millilitres of the buffered medium given above, were placed in a fluorometric cuvette. After a five minute incubation at 25° C., the cuvette was placed in the fluorometer, and the instrument zero was set. 0.2 millilitres of 0.2  $\mu$ M NADH, 2.0  $\mu$ M phosphoenol pyruvic acid, and 2.0  $\mu$ M ATP in the buffered medium was then added, the solution was gently mixed, and the fluorescence was measured until complete (two to three minutes).

Ethylene was added as a solution in the buffered medium used, at a final concentration of 100 parts per million. Incubation experiments with ethylene were done at 25° C.

The decrease in fluorescence of NADH with time is a function of the ATPase concentration because sufficient substrate, pyruvate kinase, and lactic dehydrogenase are present.



### Measurement of mitochondrial protein

A modified biuret procedure (Gornall, Bardawill, and Davies 1949) was used to measure the protein content of the particulate fraction. The method was standardized with bovine serum albumin (BSA) (Calbiochem), and the nitrogen content of the bovine serum albumin was determined by a Kjeldahl method (Ogg 1960).

One millilitre of the solution to be analyzed was added to four millilitres of biuret reagent, mixed thoroughly, and allowed to stand for thirty minutes at room temperature. A portion of this solution was transferred to a one centimeter quartz cuvette, and read against a blank containing one millilitre of distilled water, and four millilitres of biuret reagent contained in a matched one centimeter quartz cuvette, at 550 mμ, with a Beckman model DU-2.

A standard curve was prepared for each set of biuret analyses with a 0.2 % solution of BSA.

The biuret reagent consisted of 1.50 grams of copper sulfate ( $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ ), and 6.0 grams of sodium potassium tartarate ( $\text{NaKC}_4\text{H}_4\text{O}_6 \cdot 4\text{H}_2\text{O}$ ) dissolved in 500 millilitres of distilled water. To this solution, 300 millilitres of 10 % sodium hydroxide was added, after which the solution was made to one litre. One gram of





potassium iodide was added as a preservative as suggested (Gornall et al 1949). The biuret reagent was stored in brown glass bottles, and dispensed with a semi-automatic burette.

### Fluorometric analysis of the action of cellulase

The compound 8-anilino-1-naphthalene sulfonic acid (ANS) fluoresces under ultraviolet light when it combines with negatively charged groups in the presence of protein. The conjugate fluoresces most strongly when excited by light of wavelength 346 m $\mu$  (Weber, and Laurence 1954). Washed yeast cells exhibited no appreciable fluorescence when added to a solution containing ANS, indicating the lack of groups with which this chemical could react. Similar results have been found with bacterial cells (Newton 1954). Thus, changes in permeability with time can be measured by the increase in fluorescence, as ANS penetrates to the protein in the interior of the cell.

The ANS sodium salt (Eastman Kodak Co., Rochester, New York) was purified by the method described by Malcolm (1967), that is, by precipitation with ammonium sulfate, and crystallization.

Fluorescence was measured with a Turner Model III



fluorometer (G. K. Turner Associates, Palo Alto, California), fitted with a #110-811 filter (exciting filter, 350 mμ), and a 110-817 filter (recording filter, passes light above 470 mμ).

The procedure followed was that of Malcolm (1967). The fluorescence was described as a percentage of the fluorescence given off by a sample of yeast cells, plus 0.5 % cellulase, killed by boiling for five minutes, compared to a similar sample to which the cellulase has just been added. A concentration of 50 μmoles per millilitre of ANS was used. Samples from the cellulase treated yeast suspension were removed at intervals, ANS, dissolved in the medium in use, was added, and the mixture was immediately placed in the fluorometer. Three minutes were allowed for fluorescence development.

To test the linearity of the reaction between ANS, and protein, BSA, at varying concentrations, was used.

#### Measurement of the concentration of ethylene in solution

As the solubility of ethylene has been given only in water (Hodgman et al 1966, McAuliffe 1966), it was felt that analysis of the ethylene content of the buffered media used in this project was required.





McAuliffe (1966) has established a simple procedure for measurement of the solubilities of hydrocarbons in water, and his procedure was modified for use in this laboratory.

Modification of a Perkin-Elmer Model 810 flame ionization gas chromatograph to accept external columns made this analysis quite simple. An interior glass column of internal diameter of six millimeters containing one foot of aluminum oxide (Fisher Scientific Co., ltd.) was prepared, and dried under vacuum. A curved external column was prepared in a similar manner, and is shown in figure 1. Glass to glass connections on the exterior column were made with thick walled tygon tubing. The carrier gas could either be directed through the interior column, or through the exterior column, and then into the interior column, by means of a two way valve.

100 microlitres of a solution containing ethylene was injected by a Hamilton microlitre syringe through a silicone plastic septum into the upper part of the exterior column, which was disconnected from the carrier gas flow. The column was then opened to gas flow to flush the solution through the column. The liquid was stopped by the alumina, and the gas contained in it passed through to the detector.



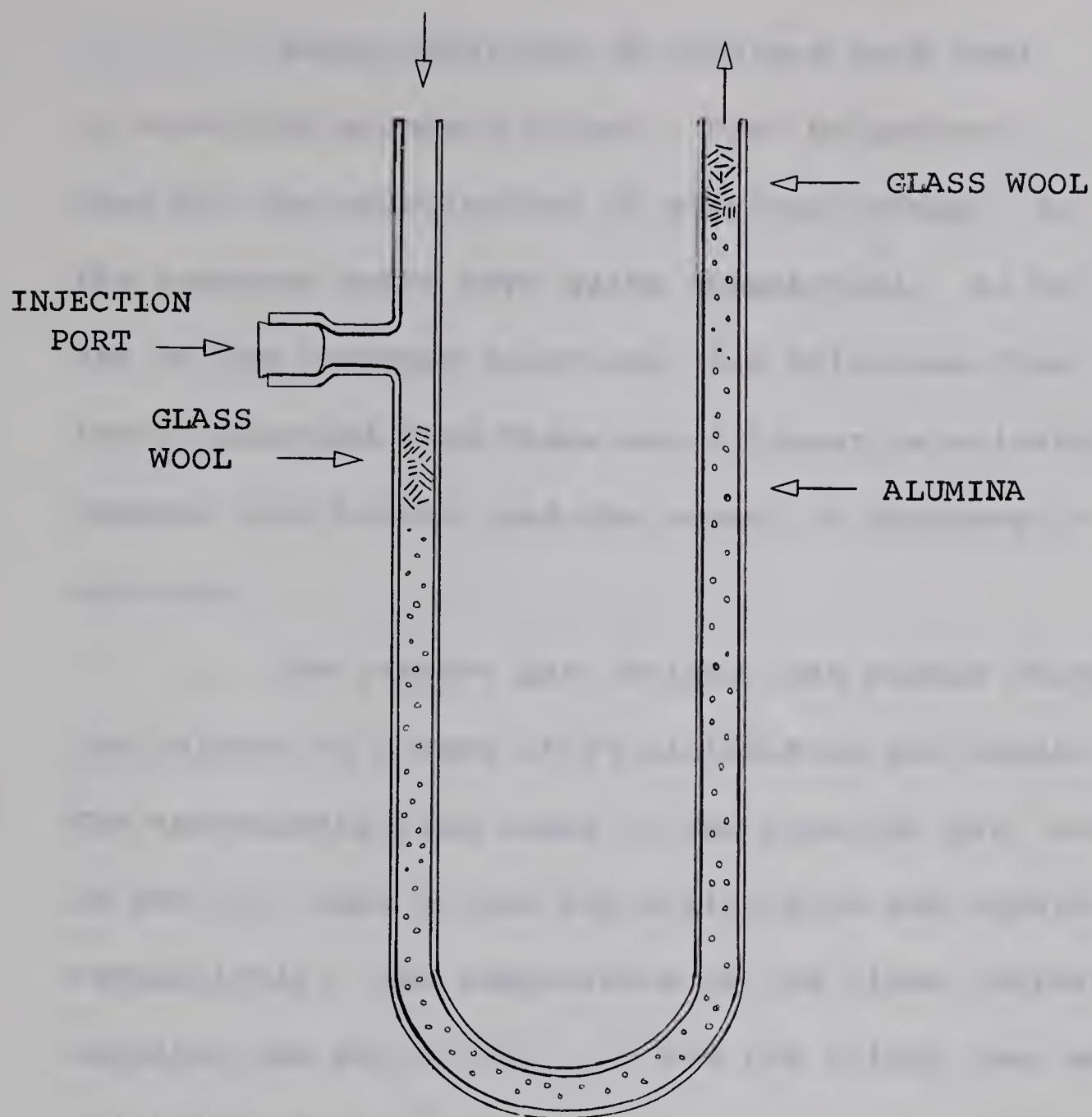


Figure 1. Diagram of the exterior column used to trap liquid in which ethylene had been dissolved. The column was constructed of six millimetre (inside diameter) glass tubing, and is shown at approximately actual size.





Water solutions of ethylene were used to establish standard curves. Peak height was used for the calculations of ethylene content, as the recorder peaks were quite symmetrical. As well, use of the standard solutions, and dilutions from them, indicated that there was a linear relationship between peak height, and the amount of ethylene in solution.

The carrier gas, helium, was passed through the columns at a rate of 35 millilitres per minute. The approximate flow rates of the hydrogen gas, and of the air, were 30 and 400 millilitres per minute, respectively. The temperature of the flame ionization detector was set at  $150^{\circ}\text{C}$ , and the column oven was maintained at  $100^{\circ}\text{C}$ .



Spectra of ethylene in solution

Ultra-violet spectra of solutions containing various concentrations of the components used in the mitochondrial preparation, suspending, and assay media were determined. Ethylene saturated samples of these solutions were compared to a blank of water, to samples not containing ethylene, and samples containing ethylene, but none of the components of the media. The absorption spectra were measured with a Beckman Model DK-1 double beam recording spectrophotometer, utilizing sealed one centimeter quartz cuvettes. All analyses were done at room temperature (Approximately 23° C.)

Infra-red spectra were determined in the same manner, employing one millimeter disposable silver chloride cuvettes, with a Perkin-Elmer Model 21 double beam recording spectrophotometer. The cuvettes were obtained from Research and Industrial Instruments Co., London, England.





Computer Simulation of the effect of ethylene on  
cellular metabolism

Early evidence obtained in this project, and that suggested by Lyons, and Pratt (1964) indicated that the utilization of ATP and ADP was influenced by ethylene. The effect upon mitochondrial, and cellular respiration could only be determined by study of a model system simulated on a computer. Such models must obviously be fairly simple, if reasonable computational times are to be achieved. One must also assume that the law of mass action holds in all mechanisms studied. As well, membrane permeability must be surmised to be representable by simple chemical equations in reversible equilibrium.

A variety of mechanisms for oxidative phosphorylation were derived from the literature. Rate constants, and concentrations of components were obtained in the same manner. A representation of Mitchell's chemiosmotic theory (Slater 1966), and a mechanism of oxidative phosphorylation involving high energy intermediates were used to determine what effect an increased concentration of ADP would have on mitochondrial oxidative phosphorylation. A large



number of other mechanisms were tried, however, problems related to their physical parameters made solution difficult.

A subset of Fortran IV was used as the basic programming language, although the graph routine was written in MAP. A very large amount of time was required to produce a program of sufficient speed, and capability to handle large parameters without error.

The regular simulator program is based in part on a program obtained from Dr. D. Garfinkel, of the Johnson Research Foundation, University of Pennsylvania, Philadelphia.

The programs were run on an International Business Machines Co. Model 7040 computer, with a model 1401 printer.





## RESULTS AND DISCUSSION

### Measurement of protein

The Kjeldahl method (Ogg 1960) was used primarily to determine the total nitrogen content of the bovine serum albumin used as a standard in the biuret tests, and not of the particulate fractions, because of the amount of time required for analysis, the size of sample necessary for accurate results, and the interference from nitrogen containing buffer components (Figure 2.).

The biuret procedure was found to be rapid, and simple to use. Figure 3 indicates the relationship between absorbance and bovine serum albumin concentration. At higher concentrations of protein, the absorbance curve was found to level off, indicating insufficient quantities of the complexing agent in the biuret reagent. As the normal levels of assay (0.5 to 5.0 milligrams of protein per millilitre) were far below this level, no changes were made in the reagent.

Figure 4 indicates the relationship between absorbance, and various dilutions of a mitochondrial suspension added to the biuret reagent. As well, measurements of the absorbance at 520 m $\mu$  (as used in



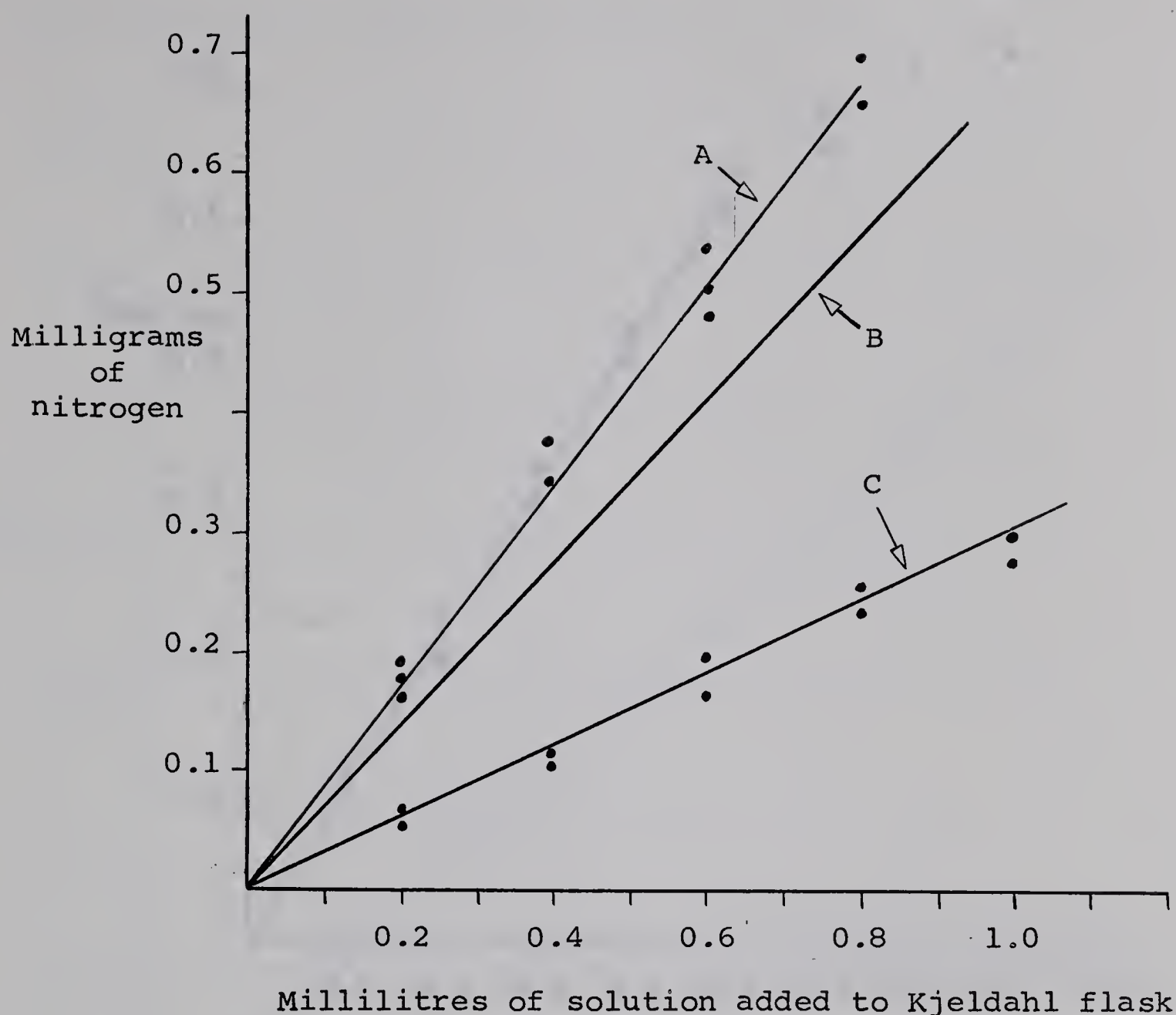


Figure 2. Calibration curves relating total nitrogen content to the volume of solution added. (Volumes of solution as given were added to Kjeldahl flasks, and dried prior to digestion.) Curve C indicates the change in N content with the change in volume of a 0.2 % BSA solution. Curve A gives the N content versus volume of a bean mitochondrial suspension in 0.50 M mannitol, 0.05 M Tris-HCl, and 0.001 M EDTA . Curve B relates the contribution of Tris to the N content of the mitochondrial suspension (calculated) .





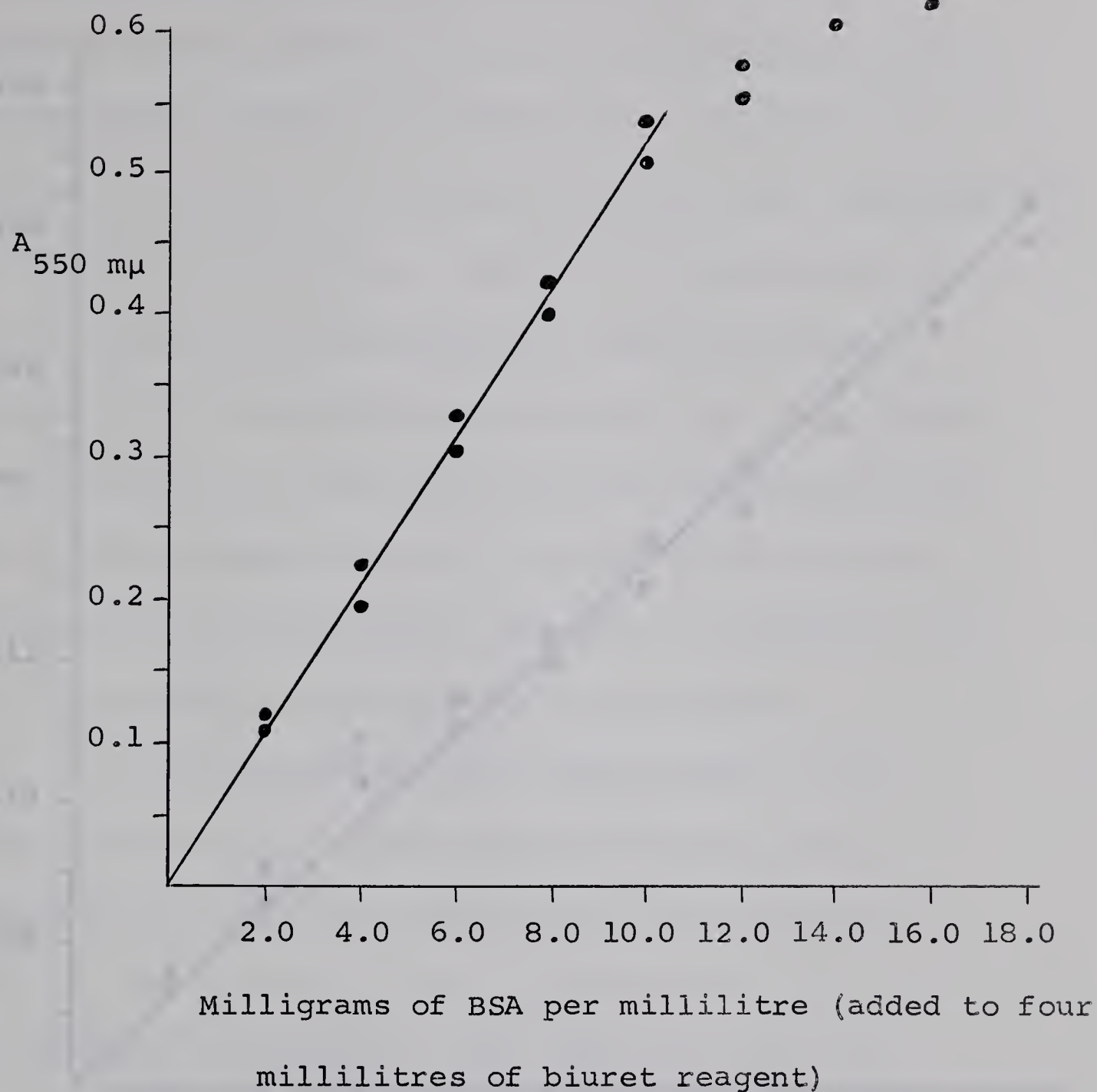
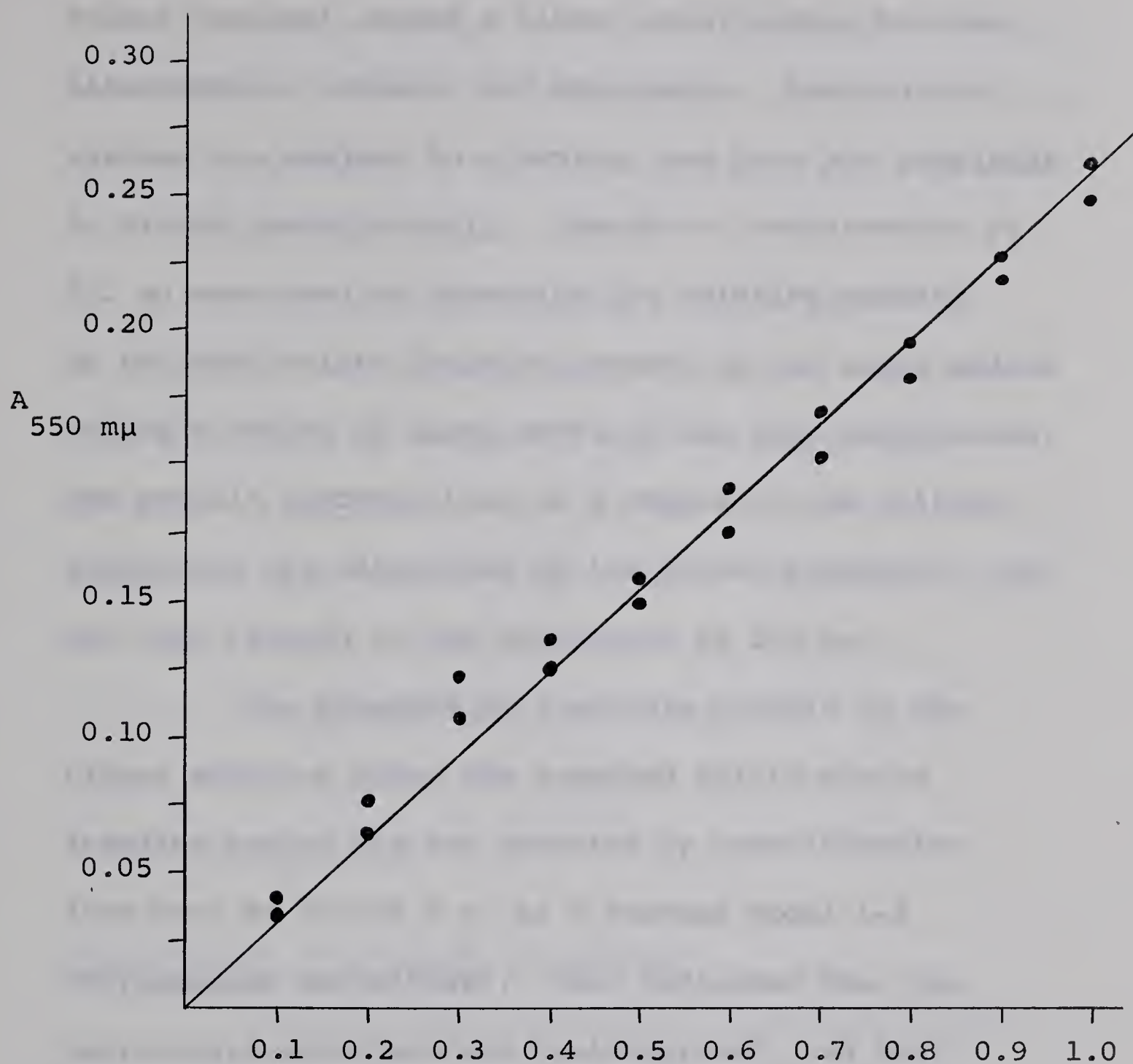


Figure 3. Calibration curve relating the absorbance of the biuret reagent at 550 mμ to the concentration of bovine serum albumin in one millilitre of water.





Millilitres of mitochondrial suspension added in  
a final volume of one millilitre to four millilitres  
of biuret reagent.

Figure 4. Relationship between the absorbance of the biuret reagent at 550 mμ, and the volume of a mitochondrial suspension.





the turbidimetric method for measuring mitochondrial volume changes) showed a linear relationship between mitochondrial content, and absorbance. Particulate systems are subject to clumping, and thus are difficult to divide quantitatively. Therefore, measurements at 520 m $\mu$  were used to determine the relative amounts of the particulate fraction present in the assay medium during a series of experiments on the same preparation. The protein concentration of a sample of the initial suspension was determined by the biuret procedure, and was then related to the absorbance at 520 m $\mu$ .

The presence of insoluble protein in the biuret solution after the standard thirty minute standing period was not detected by centrifugation (one hour at 30,000 x g, in a Beckman Model L-2 refrigerated centrifuge). This indicated that the particulate structure was disintegrated, and that its components had dissolved in the concentrated sodium hydroxide of the biuret reagent.



### Measurement of inorganic phosphate

Figure 5 shows a typical relationship between the content of inorganic phosphate in the test solutions, and the absorbance at 313 mμ. The curve was linear in the concentration region studied.

ADP, ATP, and phosphate were found to be stable in the isobutanol-benzene reagent. However, a slow hydrolysis of ATP was shown to occur after addition of the protein precipitant to the mitochondrial suspension that was to be analyzed. (Mozersky, Pettanti, and Kolman (1966) indicated that this hydrolysis occurred at approximately four percent per hour.) Rapid extraction into the organic solvent was therefore practised.

### Measurement of ethylene solubility

From the data presented by McAuliffe (1966), one would expect 131 grams of ethylene to be soluble in one million grams of water at 25° C., and 760 mm. Hg. This is equivalent to 4.79 moles of ethylene per million grams of water, or 11.5 microlitres of ethylene per 100 microlitres of water at 25° C., and 760 mm. Hg.

Analysis by gas chromatograph indicated a linear relationship between peak height, and the amount of





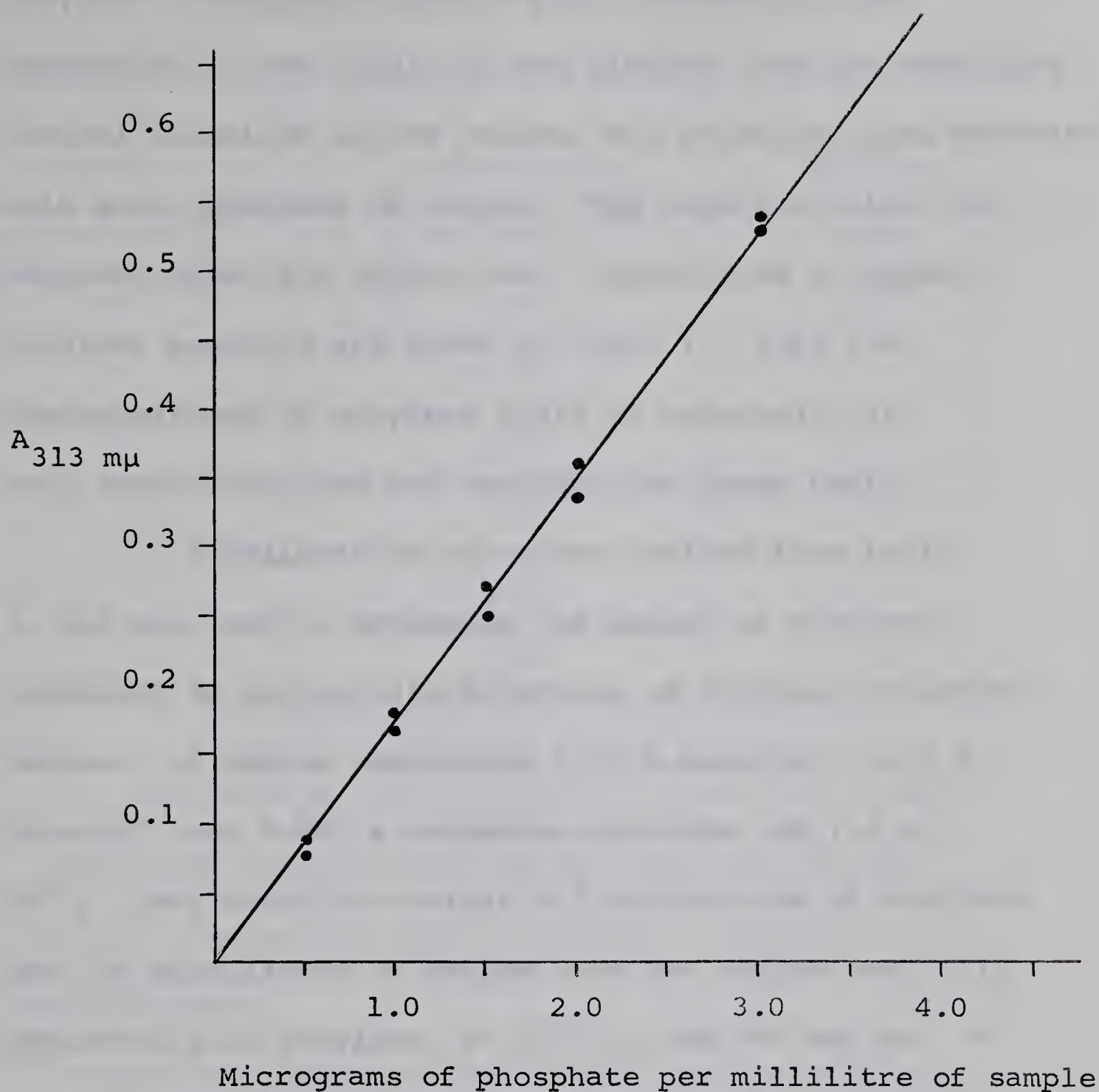


Figure 5. Calibration curve relating the absorbance of the phosphomolybdate complex at 313 mμ to the concentration of phosphate per millilitre (of the three millilitre sample analyzed by the method of Mozersky et al (1966)).



ethylene dissolved in the sample. Because of the absorption of the liquid by the alumina, and the resulting channel formation in the column, the retention time decreased with each injection of liquid. The exterior column was replaced after six injections. Values from a typical ethylene analysis are given in table 1. Very low concentrations of ethylene could be detected, but such sensitivity was not required for these tests.

A calibration curve was derived from table 1, and was used to determine the amount of ethylene contained in appropriate dilutions of ethylene saturated medium. A medium containing 0.50 M mannitol, 0.01 M Tris-HCl, and 0.001 M magnesium chloride, pH 7.4 at 25° C., was found to contain 9.5 microlitres of ethylene per 100 microlitres of medium when the medium was fully saturated with ethylene, at 25° C., and 760 mm. Hg. A medium containing 0.15 M potassium chloride, instead of mannitol, in the above medium, was found to contain 7.3 microlitres under the same conditions.

Dilutions for experimental use were made with the appropriate medium, and stored in tightly stoppered vessels.

Because of losses in gas from solution, one could not





Table 1. Determination of ethylene in solution

Percent saturation**	Peak height in inches***	Ethylene content ( $\mu$ l) ****
100	8.35	11.5
50	3.95	5.7
25	1.92	2.9
12.5	1.15	1.4
6.8	0.60	0.7
3.4	0.25	0.35

\* By a modification of the method of McAuliffe (1966).

\*\* Solutions of known saturations were obtained by diluting a saturated water solution of ethylene with distilled water.

\*\*\* Output of the gas chromatograph was recorded on a Beckman Ten Inch recorder.

\*\*\*\* Ethylene content in microlitres contained in 100 microlitres of solution of the indicated saturation, at 25° C., and 760 mm. Hg.



expect that the concentration of ethylene in the prepared media remained constant. Thus, new solutions were prepared frequently, and care was taken not to open containers holding ethylene solutions, for more than very short periods.

### Choice of tissue

The most important reasons for the choice of bean cotyledons were that they had a known history, and could be obtained at a definite stage of development. Convenience and availability also played an important role in their choice as the major source of mitochondria used in this study. Although easy to prepare, they suffered from several shortcomings. Because of the unevenness of germination, not all seedlings were of the same age. Hence, only cotyledons from plants of the same height were used. If a sufficient quantity was to be harvested in a short time, small sections of stem had to be included with the cotyledon. Since several tests showed that stem tissue contributed a negligible amount of mitochondria, this contamination was disregarded. Where possible, the primary leaf of older cotyledons was removed. Again, these leaves were found to contribute very small amounts of





mitochondria. In order to obtain 200 grams of cotyledonary tissue, larger masses of tissue were actually used in the preparation. The following amounts of tissue were actually used.

<u>Age of cotyledons</u>	<u>Weight of tissue harvested</u>
1 day	200 grams
3 days	250 grams
6 days	300 grams
10 days	300 grams

Although potatoes were easy to procure, the potato tuber was not found to be a suitable source of mitochondria. After homogenizing this tissue, and during the subsequent handlings, the homogenate turned a very dark brown. The effect of the apparent phenol oxidase activity on mitochondrial systems has not been studied, though a number of workers (Hulme, and Jones 1963, Wynn, and Fore 1965, Weinbach, and Garbus 1965, Lieberman, and Biale 1956) have indicated that most phenols and quinones are inhibitors of oxidative phosphorylation. As well, since the growing season in this locale is quite short, most farmers use chemical treatments to kill the potato foliage prior to harvest. The



type of defoliant used is often difficult to ascertain. As a result, metabolic inhibitors may be present in the tubers. The prolonged storage required because of the short growing season also meant that a physiologically uniform tuber could not be obtained throughout the year.

The bird's nest fungus Cyathus stercoreus was initially tested as a source of mitochondria, as it is known to contain a few very large mitochondria (Lu 1965). Since rupture of the mycelium was found to be very difficult its use was discontinued.

A strain of yeast (Saccharomyces cerevisiae ATCC 4111) was found to be a suitable source of mitochondria. The Nossal cell disintegrator, and extensive sonication were first used to break the yeast cell walls, but gave such low yields of mitochondria that sufficient assays could not be carried out. Sonication of cellulase treated yeast was found to give fairly large yields of mitochondria, even though the cellulase used was not primarily active towards the yeast cellulose. It is possible that it removed enough cellulose to allow rupture without damage to the yeast mitochondria. Preliminary studies were done with a brewer's yeast obtained from a local brewery, but this yeast was a heterogeneous mixture of yeast strains.





Therefore, a pure strain was used in subsequent experiments to permit duplication of the experimental material by us, and by other workers.

Rat liver is the most commonly used source of mitochondria, and much information about the characteristics of this material is readily available. The use of rat liver mitochondria for comparison purposes was therefore of particular value.

A description of the results of the search for the best preparation procedure for each type of mitochondria is given in the following sections.



Bean cotyledon mitochondria

## Preparation

Preliminary trials indicated that very careful preparation was required if intact, and non-contaminated mitochondria were to be obtained.

Low yields of mitochondria, and poor rates of respiration were found to result from as little as ten seconds of full speed homogenization in a cooled Waring blendor. This may have been caused by the vortex formed by the high speed cutting blades, with subsequent rupture of the mitochondria, and denaturation of the mitochondrial protein.

In several preliminary runs, an electrically driven meat grinder (Phillips Gourmet Center) was used. (The metal grinding chamber, which was made from a nickel coated alloy, was precooled prior to use.) Several problems were apparent. As the tissue was not actually suspended in the buffered medium, there could be little control of the pH, or osmotic concentration of the homogenate while it remained in the grinder. As well, the amount of time required to grind large volumes of tissue was excessive. A particulate fraction was obtained, after centrifugation, that showed only a





slight response to substrates. While this method is perhaps suitable to the preparation of large masses of tissue, it was not suitable for preparation of the type of particulate preparation wanted.

The use of a cooled mortar and pestle was discontinued when yields of mitochondria were found to be insufficient for the experiments to be done. This was found to be a very arduous procedure, and not reproduceable.

All of the above procedures resulted in a large amount of tissue pulverization. Filtering through cheesecloth, and centrifugation was not sufficient to remove all of this debris. Mitochondrial suspensions prepared by these methods usually contained large amounts of fibrous material, and starch granules if prepared from one, three, or six day tissue. Starch granules were not evident in preparations from ten day tissues.

A laboratory hydraulic press, with a large stainless steel ram, and stainless steel container was then tried. The procedure given in the section on methods of preparation was developed after a number of preliminary trials. It was found that the ram and container could easily be cooled, and because of their bulk, would remain at a low temperature during the crushing of the



tissue. After filtering through cheesecloth, the homogenate contained only small amounts of debris, which were easily removed by centrifugation. Up to 400 grams of tissue could be processed at one time in 800 millilitres of buffered medium, thus removing the problems of temperature control, lack of uniformity between batches of homogenate used in one mitochondrial preparation, and excessive time spent in crushing the tissue. For uniformity between preparations, a mass of two hundred grams of cotyledons was used. When more, or less tissue was used, the amount of mitochondria recovered per gram of cotyledons used was decreased.

Table 2 shows the nitrogen content of the mitochondrial preparation from the various ages of bean cotyledons studied. The decrease in the yield of mitochondria may indicate a decline in the mitochondrial content of the bean cotyledon with age, or may have been a result of the preparation procedure. It is possible that the softer tissue of 6, or 10 day bean cotyledons deformed rather than rupturing as did the younger tissues. It should be noted that the nitrogen content, on an organ basis, did not decrease, and that the difference given in table 2 may reflect only a loss of water from the cotyledons.

As the major purpose of the mitochondrial preparation was the study of mitochondrial volume changes, phosphate could not be used as the buffer component in







Table 2. Total nitrogen content of mitochondria prepared from various ages of bean cotyledons

Age of tissue (days)	Total nitrogen content (mg)*	Number of preparations**
1	7.26 ( $\pm 0.52$ )	8
3	5.43 ( $\pm 0.65$ )	12
6	4.59 ( $\pm 0.68$ )	14
10	4.05 ( $\pm 0.40$ )	5

\* The amount of nitrogen in the mitochondrial suspension (prepared in the standard manner) was determined by a Kjeldahl procedure (Ogg 1960). Values have been corrected by subtracting the nitrogen content of the medium. The range in nitrogen content between the different preparations is given in brackets.

\*\* This refers to the number of preparations used in the calculation of this data.



the preparation, or assay media, for it may induce mitochondrial swelling (Lehninger 1964). Tris was used in preliminary trials, and gave adequate pH control. Little difference was found in the rates of oxygen uptake, and the respiratory control ratios of mitochondrial preparations made in Tris, or a new buffer, Tricine. Phosphate, or histidine were used for oxygen uptake studies at pH levels below the buffering range of Tris.

0.05 M Tris-HCl, pH 7.6 at 0° C., was sufficient to maintain the pH (between pH 7.4, and 7.5 at 0° C.) of the homogenate prepared by the press method. In the medium used for suspending, and washing of the mitochondrial fractions, and in the assay medium used in the polarograph, or in the spectrophotometer, 0.01 M Tris-HCl was found to be adequate. If the mitochondrial suspension was stored at room temperature for four to five hours, a three to four unit pH drop occurred, possibly as a result of an accumulation of organic acids.

Fresh homogenates prepared in an unbuffered medium had a pH ranging from 5.4 to 6.0 at 0° C. No respiratory control, or response to substrates was shown by preparations made at pH 6.0, and at pH 6.5. A very slight amount of respiratory control was shown at pH 7.0, using phosphate as the buffer.





It would thus appear that a medium buffered at pH 7.4 at 25° C. is suitable for bean cotyledon mitochondria. Little difference was found between different ages of cotyledons. Mitochondria from a variety of sources are usually studied in media buffered at pH 7.4. While it is possible that this pH is optimum for most mitochondrial preparations, very little research has been done on the characteristics of mitochondria at other pH levels. Good et al (1966) has put forth a range of new buffering compounds, and one would expect that his initiative would provide the impetus for further investigation of this field.

0.30 M sucrose was used as the osmotic stabilizer in the buffered media used for homogenization of bean cotyledons, and for the washing and assay of the particulate fraction used in preliminary experiments. However, the addition of a concentrated mitochondrial suspension (in 0.30 M sucrose medium) to an assay medium containing the same concentration of sucrose, was found to cause a rapid swelling of the mitochondria (Figure 6.). This indicated that mitochondria contribute to the osmotic concentration of the suspension in which they were contained, and that an increase in



Addition of mitochondrial suspension

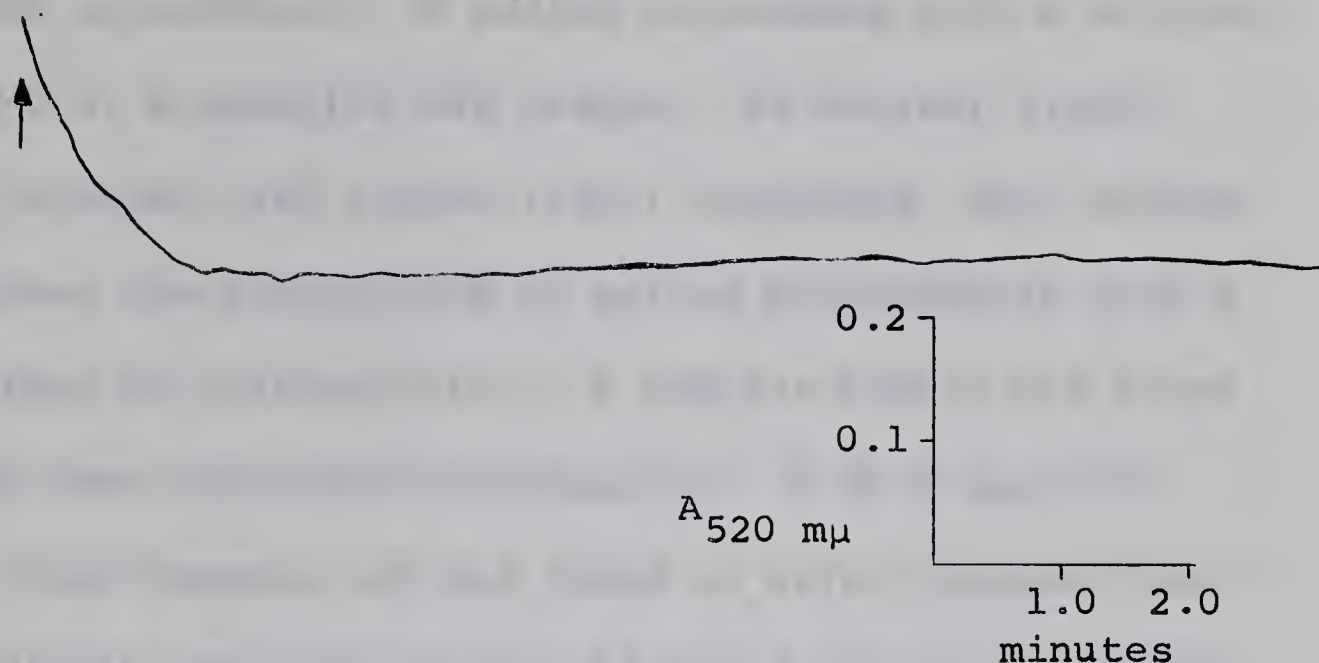


Figure 6. Three day bean cotyledon mitochondrial swelling induced by the addition of 0.20 millilitre of a concentrated mitochondrial suspension (2.0 milligrams of mitochondrial protein per millilitre) in 0.30 M sucrose, 0.01 M Tris-HCl, and 0.001 M magnesium chloride, pH 7.4 at 25° C., to 2.8 millilitres of the same buffered medium. The turbidity was measured at 520 mμ at 25° C.





concentration of the osmotic stabilizer is necessary.

During preliminary experiments with potato tuber mitochondria, a medium containing 0.25 M sucrose, and 0.37 M mannitol was tested. As Verleur (1965), and Wiskich, and Bonner (1963) suggested, this system allowed the preparation of active mitochondria with a minimum of contamination. A similar result was found with bean cotyledon mitochondria. 0.50 M mannitol was also tested, and was found to allow preparations of similar characteristics to those prepared in media containing the mixture of osmotic stabilizers. Another advantage of the use of mannitol alone is the removal of one component from the medium.

The initial swelling shown in 0.30 M sucrose (Figure 6.) was not found when media containing 0.25 M sucrose and 0.37 M mannitol, or 0.50 M mannitol were used, except when very high concentrations of mitochondria were added. The use of such concentrated mitochondrial suspensions for volume studies necessitated the use of large slit widths in the spectrophotometer if the light beam was to penetrate the suspension. A large error is introduced by the use of concentrated suspensions into measurements of changes in mitochondrial volume because



light scattering becomes excessive (Koch 1961).

Lehninger (1962) indicates that mitochondrial large amplitude volume changes are inhibited to some extent by both sucrose and mannitol. Similar results with mitochondria prepared from cauliflower heads were shown by Lyons, and Pratt (1964). 0.125 M potassium chloride was suggested as the alternative, even though a gradual spontaneous swelling occurs when it is used (Lyons, and Pratt 1964). The slow swelling is a result of the rapid penetration of the mitochondrion by the salt, or more properly, its ions. As well, Schneider (1946), and Kennedy, and Lehninger (1949) have shown that 0.125 M potassium chloride in the medium increases the amount of mitochondrial agglomeration. Lyons, and Pratt (1964) did not determine whether this had occurred in their mitochondrial preparations.

0.15 M potassium chloride was used for volume change studies because of the rapid swelling found to occur at lower concentrations. A slow spontaneous swelling was evident in all preparations tested in buffered media containing potassium chloride as the osmotic stabilizer. No agglomeration was visible in samples viewed under the phase contrast





microscope. 0.15 M potassium chloride was used only in the wash, or suspending, and assay media.

The characteristic decrease in mitochondrial volume induced by ATP, and increase in volume caused by phosphate, was shown by bean cotyledon mitochondria (prepared from one, three, and six day bean cotyledons) in potassium chloride, mannitol, and sucrose media (Figure 7.). The changes in volume were somewhat smaller in sucrose media than in the other two.

Ethylenediaminetetra-acetic acid (EDTA) was added to the homogenization, and wash media to eliminate possible inhibitions, and mitochondrial swelling caused by divalent ions released by the tissue and the stainless steel ram and container, during the homogenization of the tissue. The level of EDTA added to the media was based on the literature (Bonner 1965). The addition of bovine serum albumin, and cysteine to the homogenization medium was also based on suggestions in the literature (Hobson et al 1966, Wiskich, Young, and Biale 1964). No analysis for a requirement for EDTA, BSA, or cysteine was made.

Magnesium ions were found to stimulate respiration if added to the mitochondrial suspension after



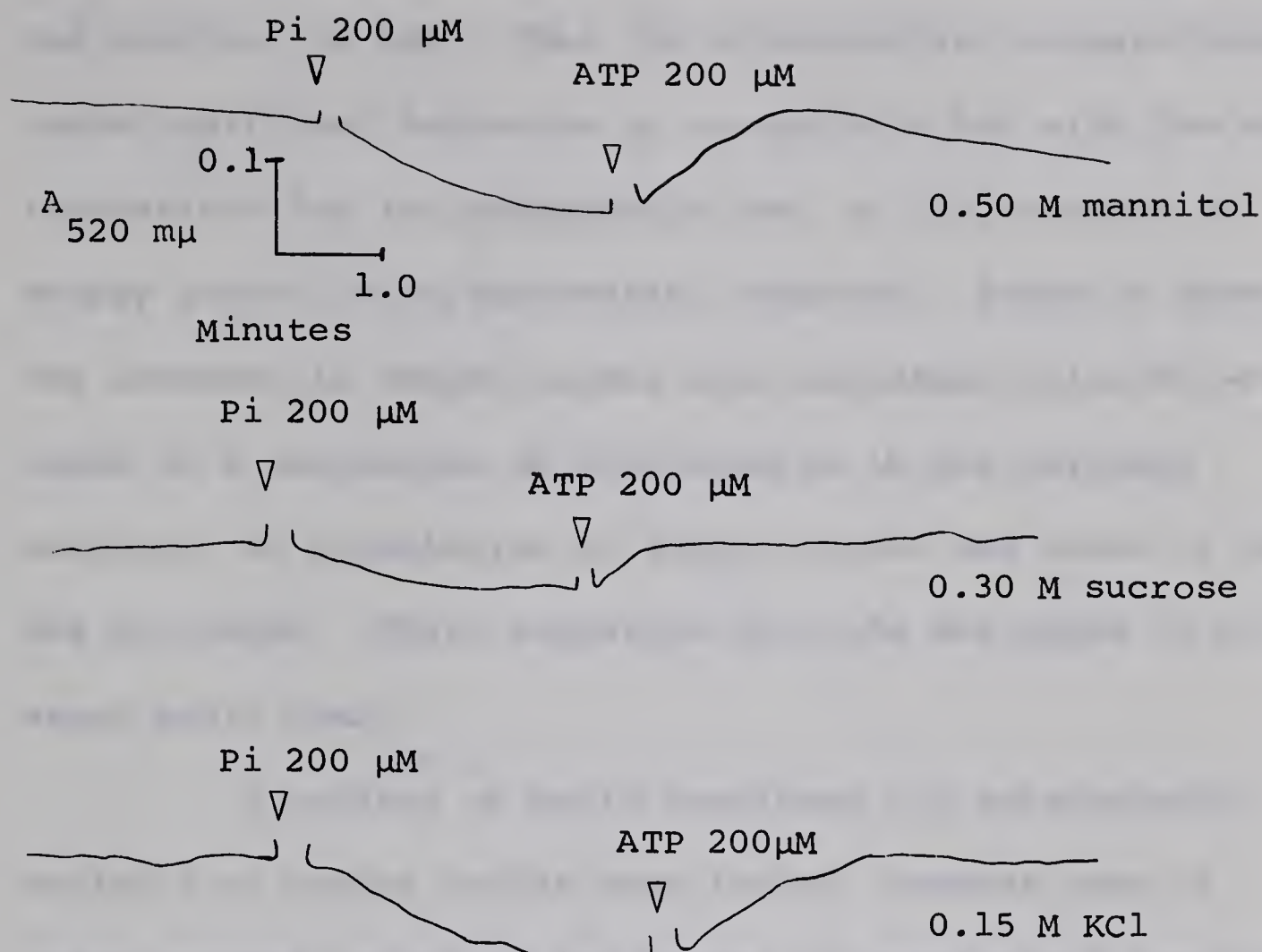


Figure 7. Volume changes induced by phosphate (Pi), and ATP in bean cotyledon mitochondria (three day) suspended in media containing the osmotic stabilizer indicated with each trace, and 0.01 M Tris-HCl, and 0.001 M magnesium chloride, pH 7.4 at 25° C. Recording was begun after the mitochondrial suspension had been added to the medium in the spectrophotometer cuvette.





the addition of ADP. Thus the mitochondrial preparations lacked sufficient magnesium to co-ordinate ADP with the enzymes responsible for its phosphorylation, or its transfer of energy across the mitochondrial membrane. Figure 8 shows the increase in oxygen uptake when magnesium chloride was added to a suspension of mitochondria in the Oxygraph cuvette. No stimulation of oxygen uptake was found if ADP was not added. Thus, magnesium chloride was added to all assay media used.

A variety of media developed for polarographic analysis of oxygen uptake were tested, however none of these systems increased the amount of respiratory control shown in the simple medium containing 0.50 M mannitol, 0.01 M Tris-HCl, and 0.001 M magnesium chloride, pH 7.4 at 0° C. The lack of phosphate in this medium was not found to limit phosphorylation, for additions of phosphate to a mitochondrial suspension increased neither respiratory control nor the rate of oxygen uptake.

In order to obtain a reasonable yield of active, and non-contaminated mitochondria, preliminary trials (in conjunction with the other preliminary experiments discussed earlier) were done to determine a satisfactory



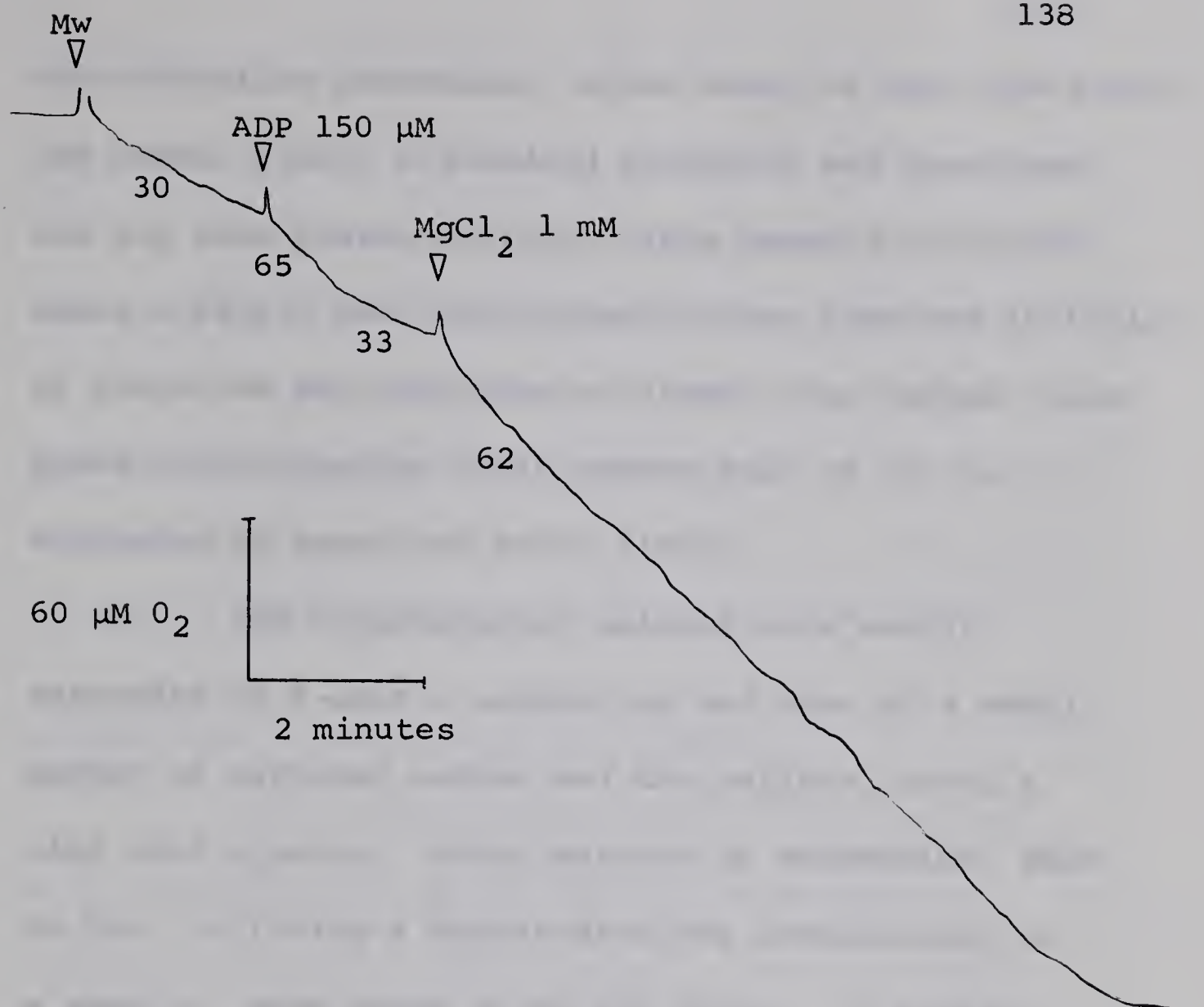


Figure 8. Polarographic trace of the oxygen uptake of three day bean cotyledon mitochondria assayed in a final volume of three millilitres of medium containing 0.50 M mannitol, 0.01 M Tris-HCl, and 0.003 M succinic acid, pH 7.4 at 25° C. 300 micrograms of mitochondrial protein were added, and the analysis was carried out at 25° C. The initial oxygen concentration was assumed to be 240 μM per litre, and the numbers below the trace indicate the rate of oxygen uptake (μMoles/minute/millilitre of suspension). Mw indicates the addition of washed mitochondria. Other additions are given as final concentrations.





centrifugation procedure. After study of the literature, and these trials, a standard procedure was developed. One day bean tissue contains large amounts of starch, hence a fairly fast centrifugation was required initially. If there was any carry-over of starch, the second "slow" speed centrifugation would remove most of it, as suggested by Lyons, and Pratt (1964).

The mitochondrial pellets were easily suspended by a gentle sucking up and down of a small amount of buffered medium and the pellets, using a wide bore pipette. Other methods of suspension, such as that utilizing a Potter-Elvehjem homogenizer, or a spatula, were found to be too harsh. Of course, severe pipetting had the same result as these methods.

For simplicity of preparation, and to obtain a degree of uniformity among mitochondrial fractions prepared from different ages of cotyledons, the same procedure was used throughout. While this method can not be expected to be optimum for all of the various ages of tissue, we felt that it was reasonably satisfactory for all.

A mass of two hundred grams of bean cotyledons, in four hundred millilitres of medium could be processed with the press, and centrifuge (International Equipment



Co. Model B-20 refrigerated centrifuge, with a # 870 rotor) in about fifty minutes.

After suspending the mitochondrial pellets, the suspension was stored in an ice-cold test tube in an ice bath, and analyses were begun immediately. Changes in respiratory characteristics during storage at this temperature will be discussed in a following section.

#### Examination of mitochondrial integrity

Phase contrast microscopy indicated that early preparations contained much contamination (Fibrous material, starch grains, large vesicles). More careful homogenization, and handling of the suspensions reduced the amount of debris visible in the light microscope to a negligible level.

The reaction of Janus Green B with mitochondria (Lyons, Wheaton, and Pratt 1964) was shown in early preparations after fifteen to twenty minutes at 25° C. Later, more active preparations were able to change the dye's colour in about five minutes.

The method of preparation, fixation, and staining that was developed to determine the integrity of the mitochondrial fractions by electron microscopy





did not allow identification of internal details of the mitochondria. However, intactness seemed to be distinguishable. In figure 9, an electron micrograph of a standard preparation of three day bean cotyledon mitochondria, the mitochondria appear to be spherical, with an average diameter of 0.65 microns. Some contamination is visible. Most of this is a result of the deposition of components of the mitochondrial suspending medium on the grid. Formvar coated grids treated only with filtered buffered medium showed the same type of contamination.

Mitochondria prepared from different ages of bean cotyledons had similar appearances.

Parsons, Bonner, and Verboon (1965), and Opik (1965) found that mitochondria extracted from bean tissues were spherical, as were the mitochondria shown here. This would indicate that little deformation has occurred, although it is difficult to determine how shrunken the mitochondria shown in figure 9 actually are.



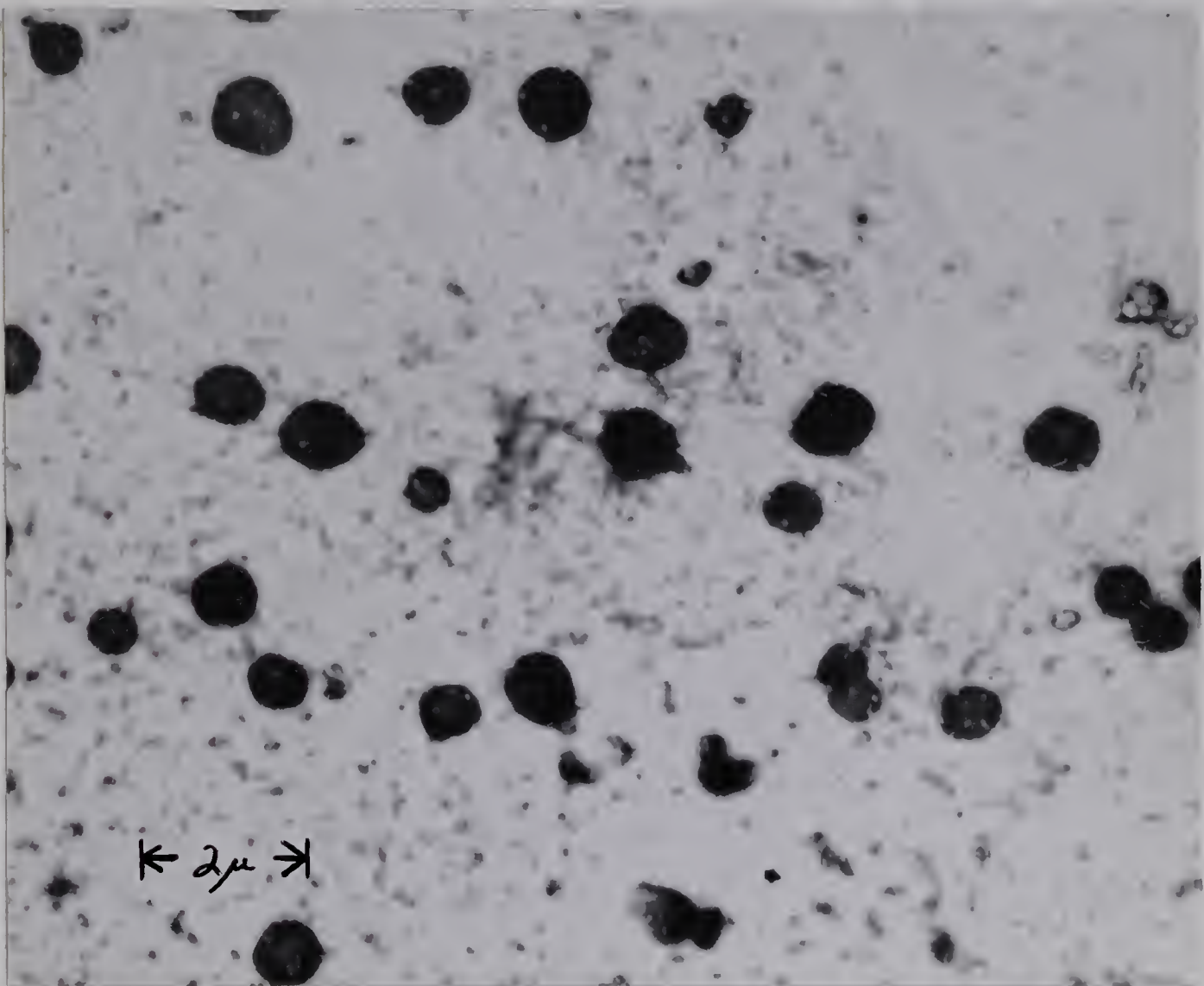


Figure 9. Electron micrograph of three day bean cotyledon mitochondria.





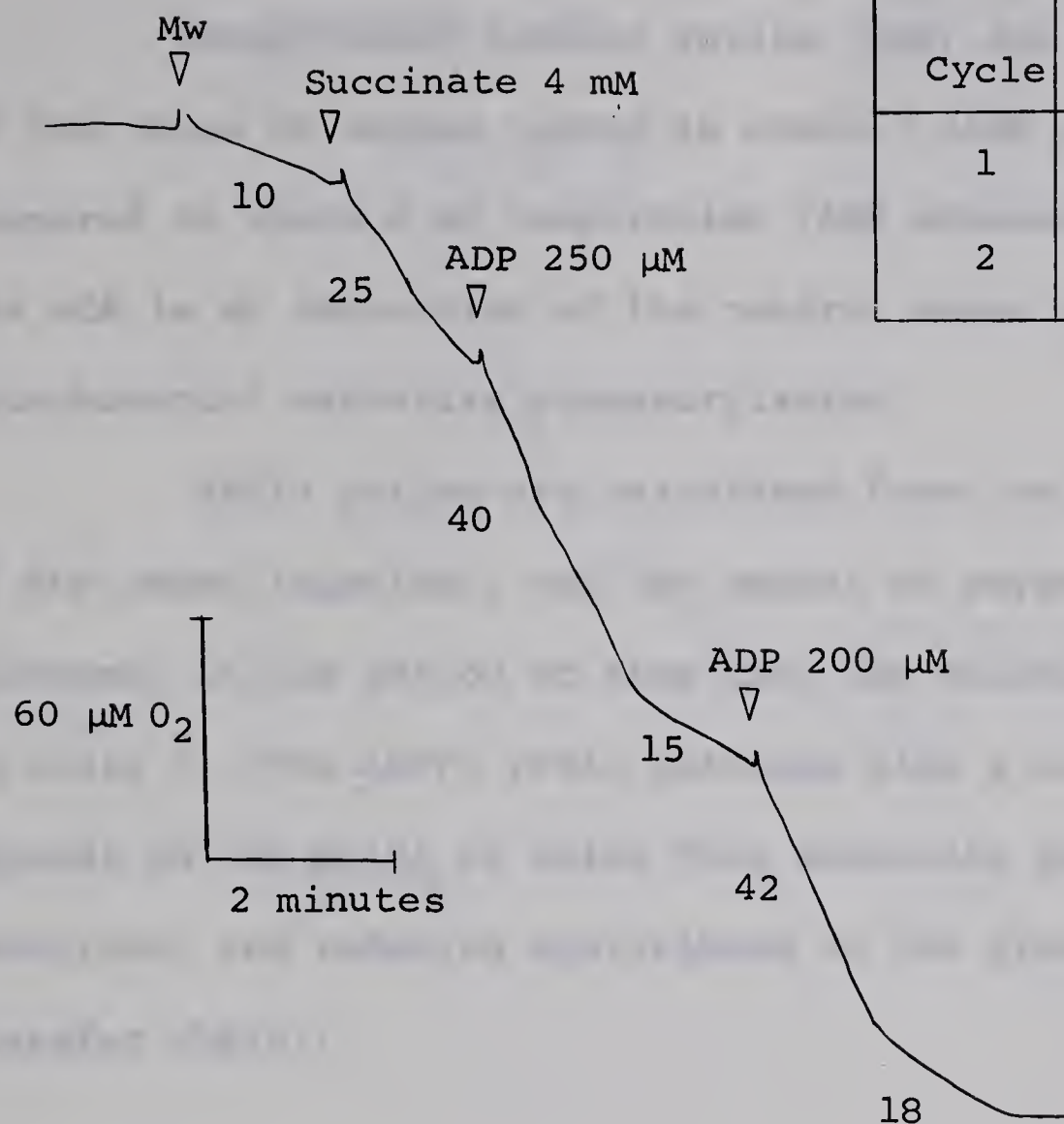
## Mitochondrial respiration

In preliminary experiments, it became evident that volume changes of bean cotyledon mitochondria were related to their respiration. Thus a polarographic oxygen electrode was constructed for use in a recording spectrophotometer. However, use of the fairly dilute suspensions of mitochondria required if reasonably accurate measurements of volume were to be made, meant that the oxygen uptake of the suspension was quite slow. Up to one-half hour was required for depletion of the oxygen present in the cuvette by some mitochondrial preparations. Most of the experiments on mitochondrial respiration were done with the Gilson Oxygraph.

The results given in figures 10 to 18, and summarized in table 3, show that the respiratory control ratios, and ADP/O ratios decreased as the tissue grew older. Glutamate gave slightly higher respiratory control ratios than succinate, or malate, but succinate was relatively more efficient as a substrate (the observed ADP/O ratios more closely approached the theoretical ADP/O ratios).

The interpretation of these results depends primarily on whether or not the preparation procedure





Cycle	RCR	ADP/O
1	2.7	1.9
2	2.4	1.9

Figure 10. Polarographic trace of the oxygen uptake of mitochondria extracted from one day bean cotyledons, assayed in a final volume of three millilitres of medium containing 0.50 M mannitol, 0.01 M Tris-HCl, and 0.001 M magnesium chloride, pH 7.4 at 25° C. 350 micrograms of mitochondrial protein were added per millilitre of assay medium, and the analysis was carried out at 25° C., using the Gilson Oxygraph. The numbers under the traces indicate the amount of oxygen uptake in  $\mu$ moles/minute per millilitre of suspension.





(Respiratory control ratios (RCR) are the ratios of the rates of oxygen uptake in state 3 (ADP present), compared to state 4 of respiration (ADP exhausted).

The RCR is an indication of the control shown by ADP over mitochondrial oxidative phosphorylation.

ADP/O ratios are calculated from the amount of ADP added ( $\mu$ moles), and the amount of oxygen uptake ( $\mu$ atoms) in the period of time that the mitochondria are in state 3. The ADP/O ratio obtained with a certain substrate depends on the point at which that substrate passes its electrons, and reducing equivalents to the electron transfer chain.)



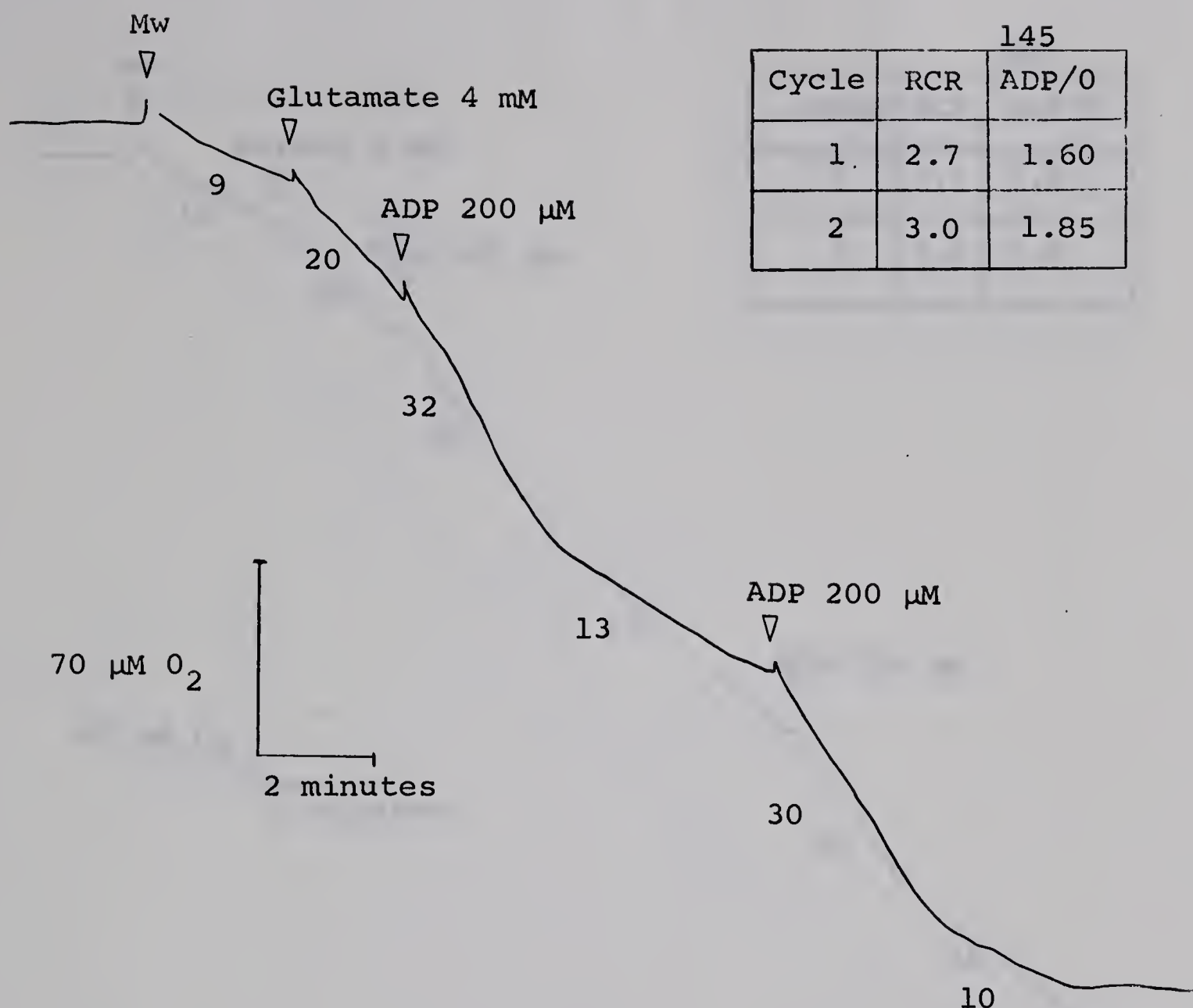


Figure 11. Polarographic trace of the oxygen uptake of mitochondria prepared from one day bean cotyledons, and assayed in a final volume of three millilitres of medium containing 0.50 M mannitol, 0.01 M Tris-HCl, and 0.001 M magnesium chloride, pH 7.4 at 25° C. 300 micrograms of mitochondrial protein were added per millilitre of assay medium, and the analysis was carried out at 25° C., using the Gilson Oxygraph. The numbers under the trace indicate the amount of oxygen uptake in  $\mu$ moles of oxygen per minute per millilitre of suspension.





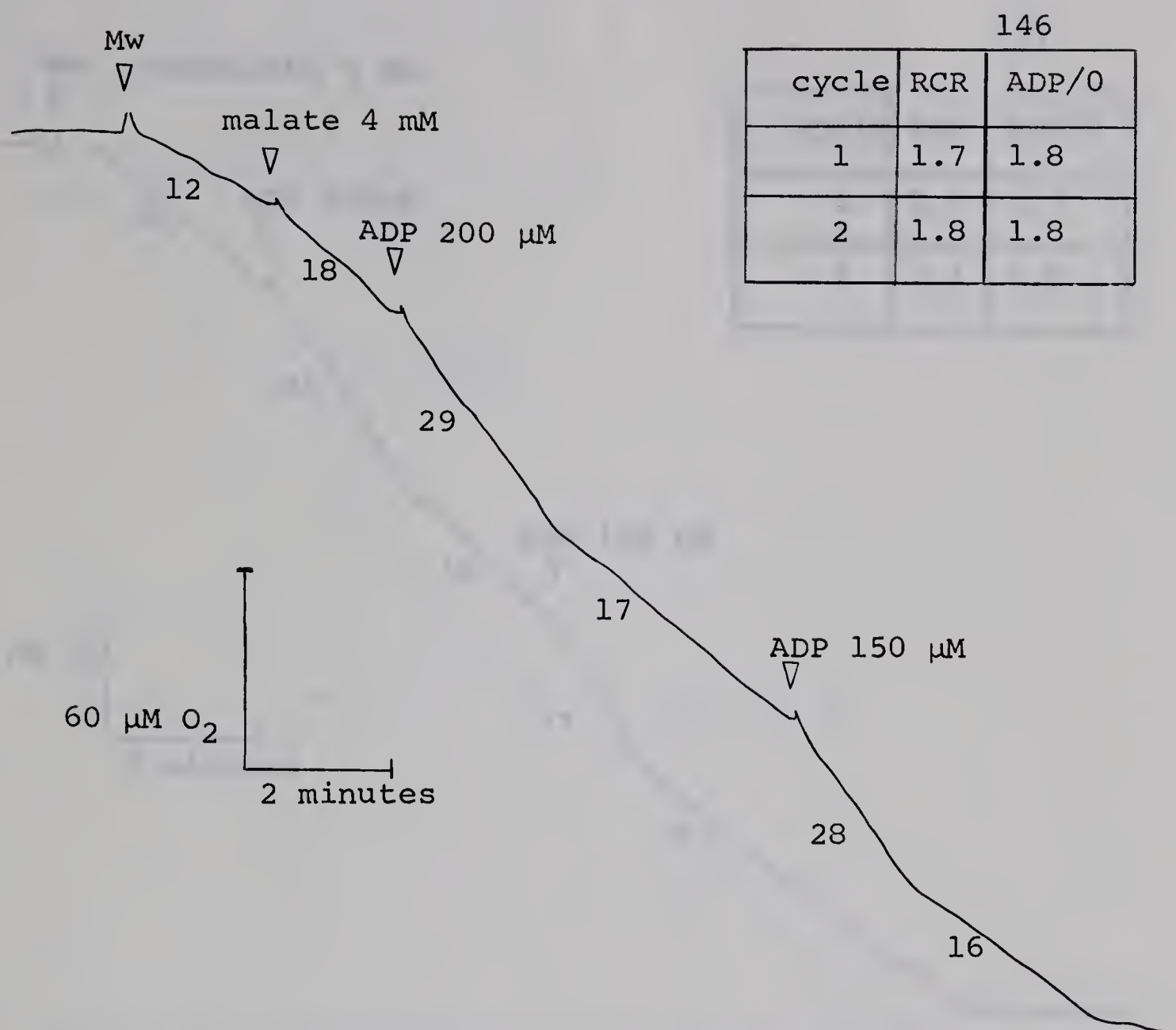


Figure 12. Polarographic trace of the oxygen uptake of mitochondria prepared from one day bean cotyledons, and assayed in a final volume of three millilitres of the standard assay medium. 300 micrograms of mitochondrial protein were added per millilitre of assay medium. Method of assay, and description of the graph symbols are given in figure 11.



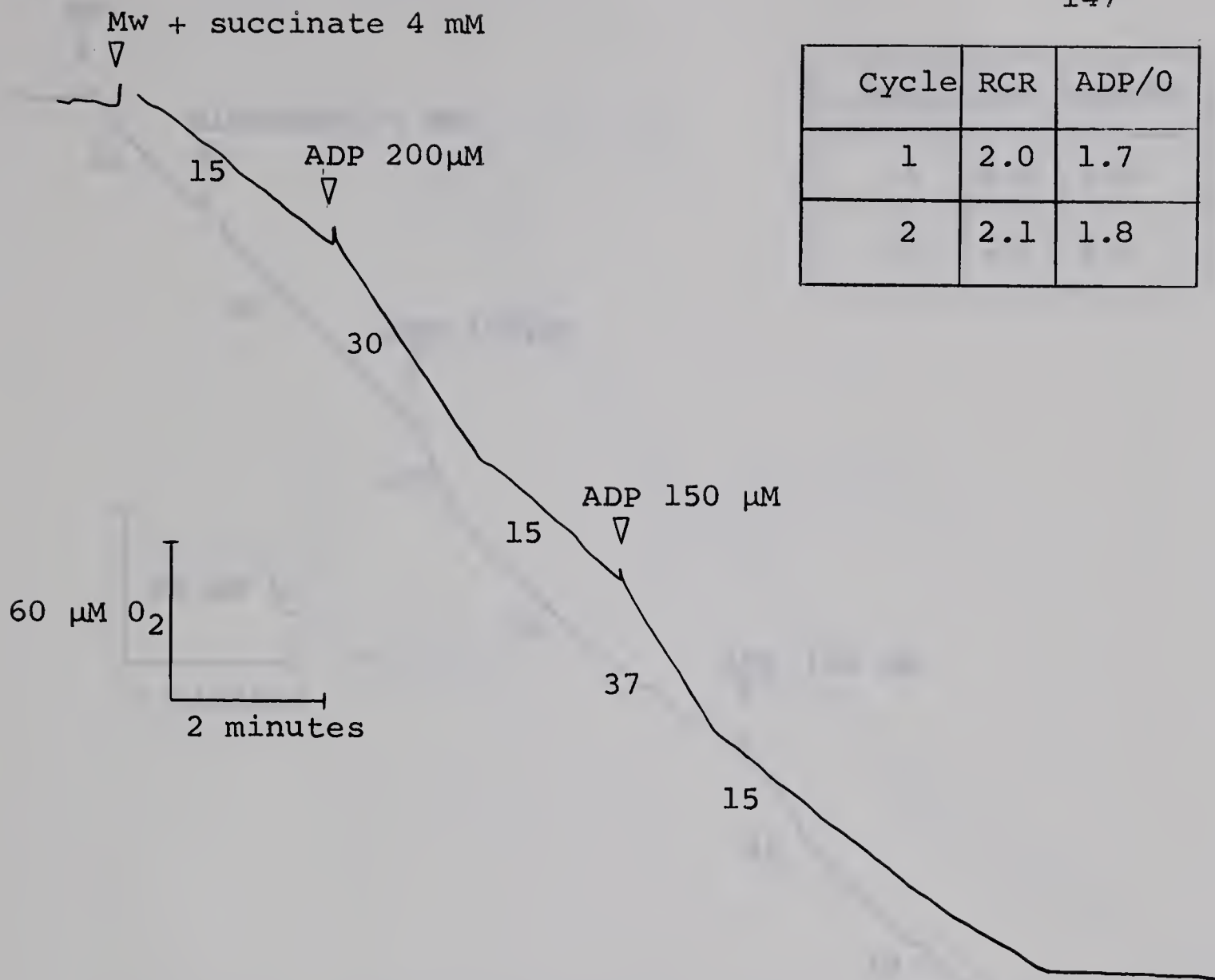


Figure 13. Polarographic trace of the oxygen uptake of mitochondria prepared from three day bean cotyledons, and assayed in a final volume of three millilitres of the standard assay medium. 300 micrograms of mitochondrial protein were added per millilitre of assay medium. Method of assay, and description of the graph symbols are given in figure 11.





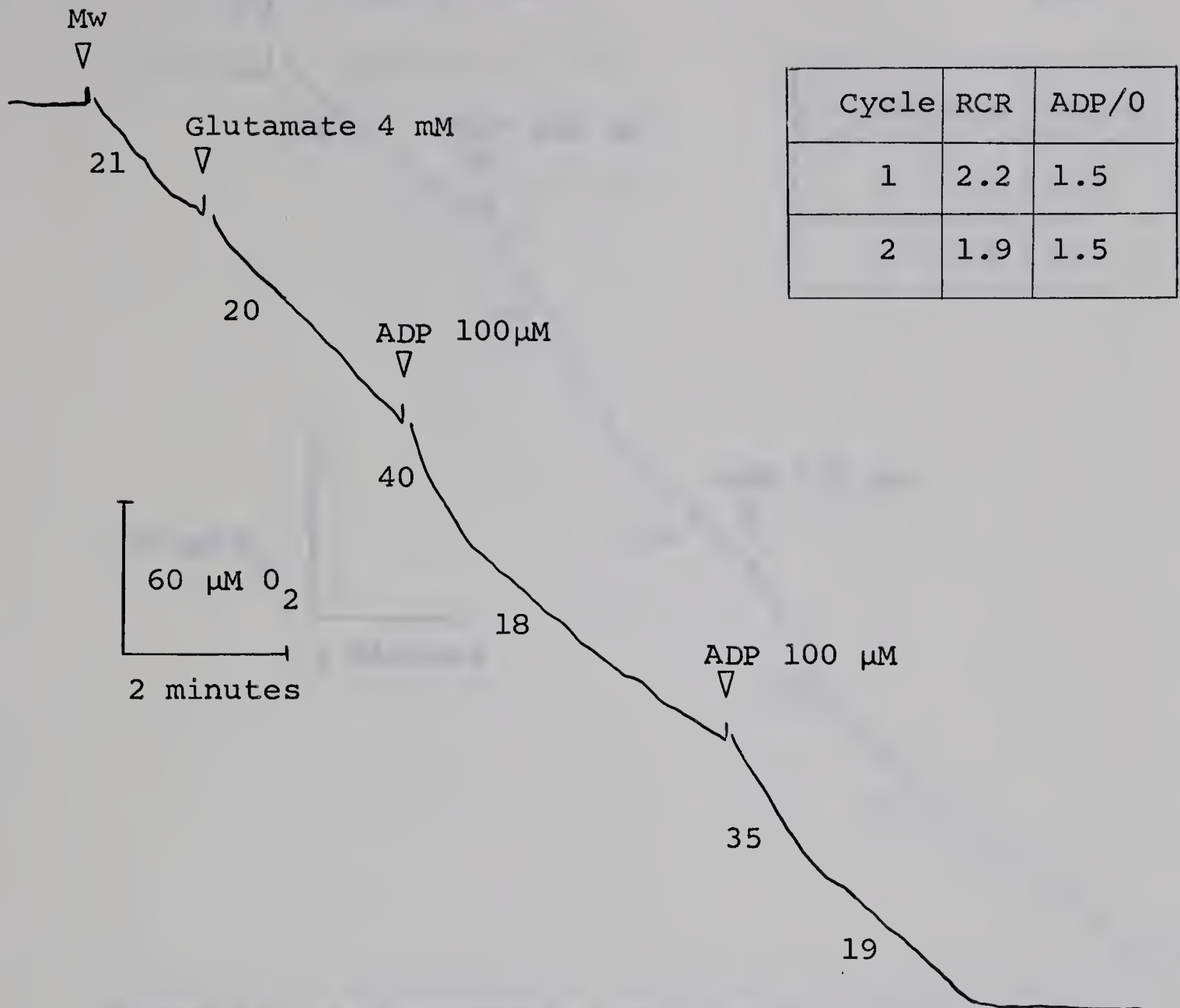


Figure 14. Polarographic trace of the oxygen uptake of mitochondria prepared from three day bean cotyledons, and assayed in a final volume of three millilitres of the standard assay medium. 300 micrograms of mitochondrial protein were added per millilitre of assay medium. Methods of assay, and description of the graph symbols are given in figure 11.



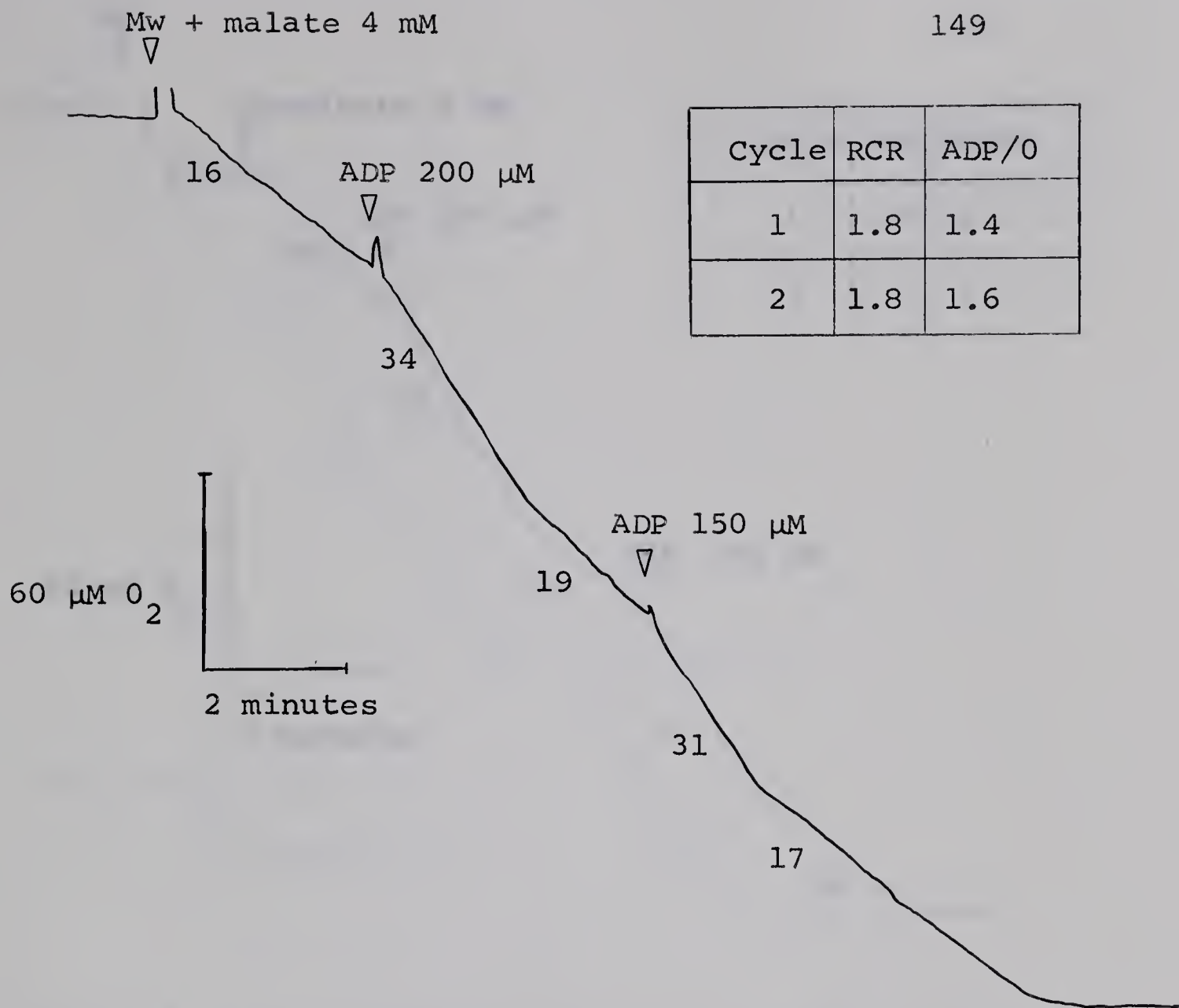


Figure 15. Polarographic trace of the oxygen uptake of mitochondria prepared from three day bean cotyledons, and assayed in a final volume of three millilitres of the standard assay medium. 300 micrograms of mitochondrial protein were added per millilitre of assay medium. Methods of assay, and description of the graph symbols are given in figure 11.





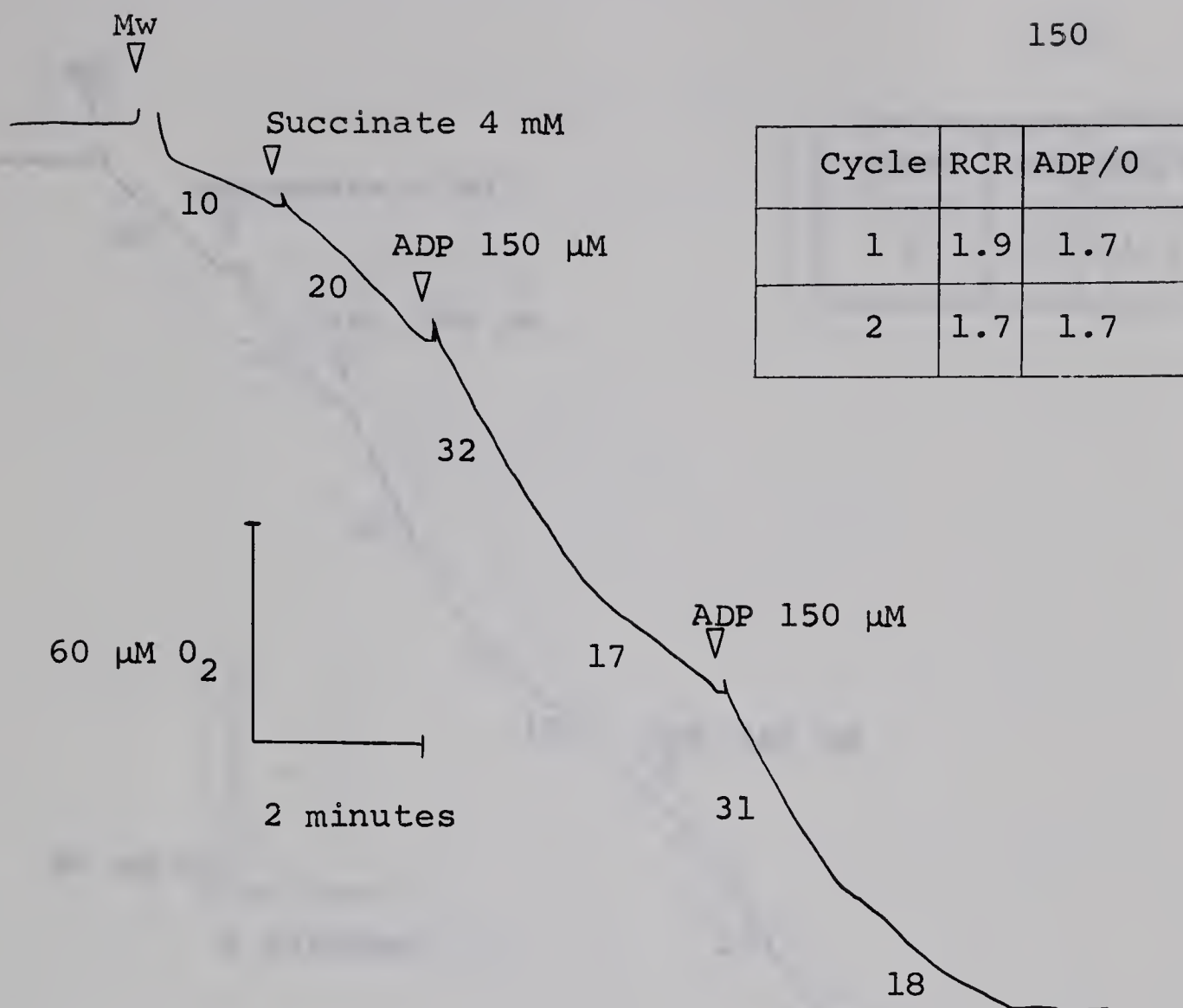


Figure 16. Polarographic trace of the oxygen uptake of mitochondria prepared from six day bean cotyledons, and assayed in a final volume of three millilitres of the standard assay medium. 300 micrograms of mitochondrial protein was added per millilitre of assay medium. Method of assay, and description of the graph symbols are given in figure 11.



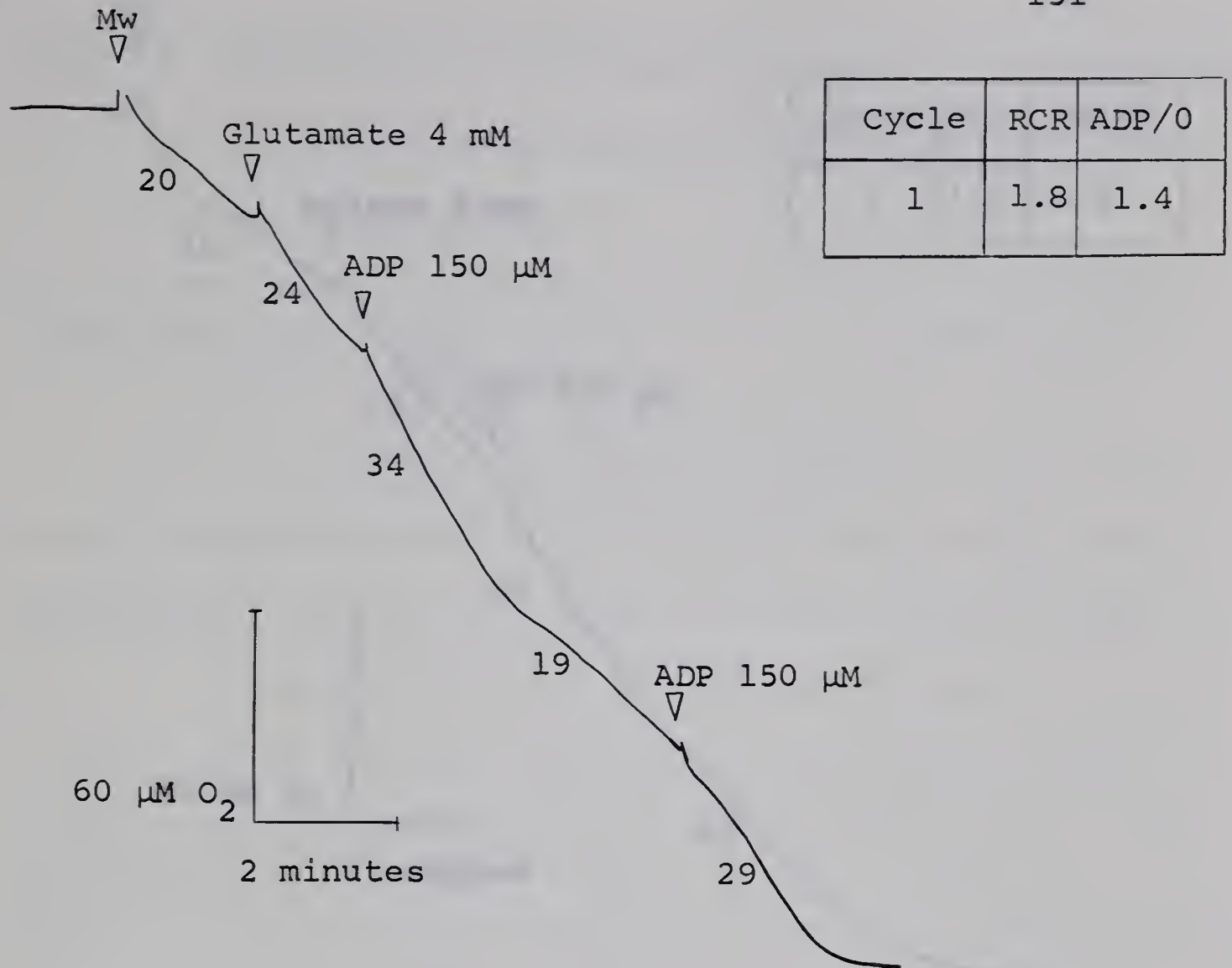


Figure 17. Polarographic trace of the oxygen uptake of mitochondria prepared from six day bean cotyledons, and assayed in a final volume of three millilitres of the standard assay medium. 300 micrograms of mitochondrial protein was added per millilitre of assay medium. Method of assay, and description of the graph symbols are given in figure 11.





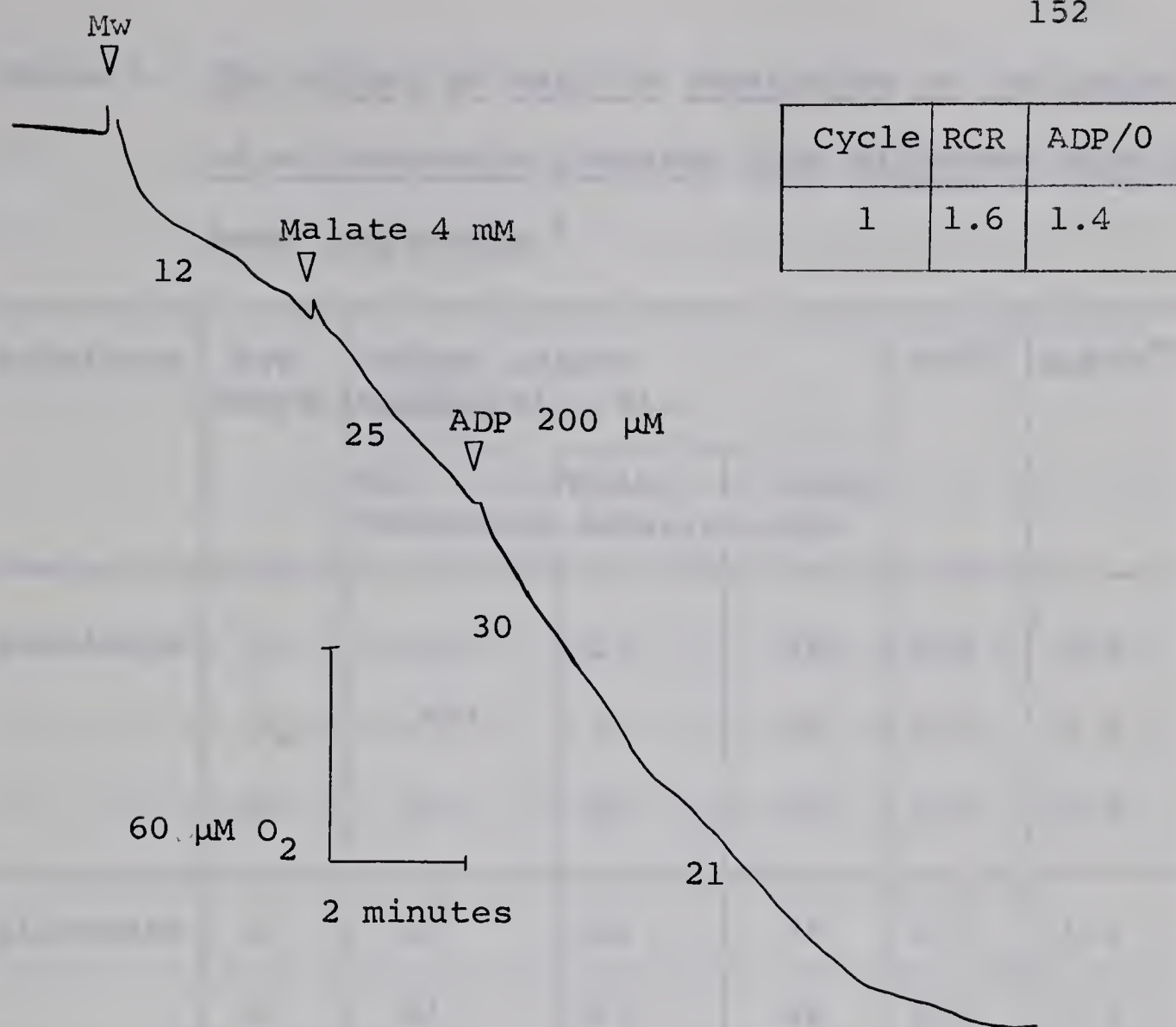


Figure 18. Polarographic trace of the oxygen uptake of mitochondria prepared from six day bean cotyledons, and assayed in a final volume of three millilitres of the standard assay medium. 300 micrograms of mitochondrial protein was added per millilitre of assay medium.

Method of assay, and description of the graph symbols are given in figure 11.



Table 3. The effect of various substrates on the properties of mitochondria prepared from different ages of bean cotyledons.\*

Substrate	Age (days)	Oxygen uptake (mmoles/min./ml.)			RCR**	ADP/O**
		No substrate	With substrate	With ADP		
Succinate	1	11	23	38	2.5	1.9
	3	—***	17	33	2.0	1.8
	6	11	22	37	1.7	1.7
Glutamate	1	10	22	35	2.7	1.6
	3	23	22	44	2.0	1.5
	6	22	26	37	1.8	1.4
Malate	1	13	20	32	1.7	1.7
	3	—***	18	37	1.8	1.5
	6	13	28	33	1.4	1.6

\* Summary of data given in figures 10 to 18.

\*\* Average of results from each trace.

\*\*\* Substrate was added to medium prior to the addition of the mitochondrial suspension.





selectively damages mitochondria from older cotyledons. Bean cotyledons have started to shrivel by the sixth day, and the less turgid condition may make breakage of the cells more difficult as they might deform rather than rupture under pressure.

A number of workers (Akazawa, and Beevers 1957, Hanson et al 1959, Young et al 1960) have found an increase in the mitochondrial activity during the first few days of germination of a variety of seeds.

(The mitochondrial activity was measured by the amount of oxygen taken up per unit weight of mitochondrial protein.) Opik (1965) has presented electron microscopic evidence that a decrease in mitochondrial activity occurs in the storage cells of the bean cotyledons. Five days after wetting of the seed, most of the mitochondria in the storage cells have become darkened. Opik suggested that the oxygen uptake measured by the other workers may have been primarily that of an alternative oxidase system, rather than that involved in mitochondrial oxidative phosphorylation.

A well developed mitochondrial system is present in dormant seeds, and on germination, these seeds show neither cell division nor expansion (Opik 1965).



Of course, not all of the cotyledonary tissue may undergo immediate senescent changes, and we have shown here that one can prepare mitochondria from six day cotyledons. Opik (1965) indicates an increase in lignification of the vascular bundles, and in the complexity of mitochondria in the vascular bundle cells during germination. If one does not accept Opik's oxidase theory, the contribution of the vascular bundle cell mitochondria must be fairly large to account for the oxygen uptake of the cotyledon five days after wetting of the seed, when all of the storage cells appear to be dying. It is thus possible that preparations of mitochondria from older cotyledons are derived primarily from the vascular tissues of the cotyledon.

The yield of mitochondria from one, three, and six day old cotyledons allowed a lengthy series of experiments, particularly when the polarograph-spectrophotometer combination was used. The oxygen uptake curves shown in figures 19 to 23, and summarized in table 4, are for mitochondrial fractions stored one, or three hours after the preparation had been completed, at 0° C. In all cases, there was a decrease in respiratory control, and in the ADP/O ratios, compared to the results given for mitochondrial fractions that





were analyzed immediately after preparation. It should be noted that the substrate used throughout this series of experiments was succinate. It is possible that another substrate would have been more efficient, but preliminary results indicated little difference among the substrates tested. One day bean cotyledon mitochondrial preparations were more tightly coupled than preparations from older tissue. Thus, it is possible that mitochondria from older cotyledons are less able to withstand the preparation procedure and the storage on completion of the preparation procedure.

Changes in the respiratory characteristics of the mitochondria were apparent within an hour after completion of preparation, hence storage at 0° C. did not halt degradative changes in the preparations. As a result, most experiments were done within an hour after the preparation was completed.

In a number of tests with one day old bean cotyledon mitochondria, storage at 0° C. for ten to twenty minutes resulted in an increased response to succinate or glutamate, when compared to a sample



tested immediately after the preparation procedure was finished. This may indicate that these particular preparations had an excess of endogenous substrates, and that this substrate was used up during the period of storage. There was no increase in respiratory control or ADP/O ratios shown by any of these stored preparations.





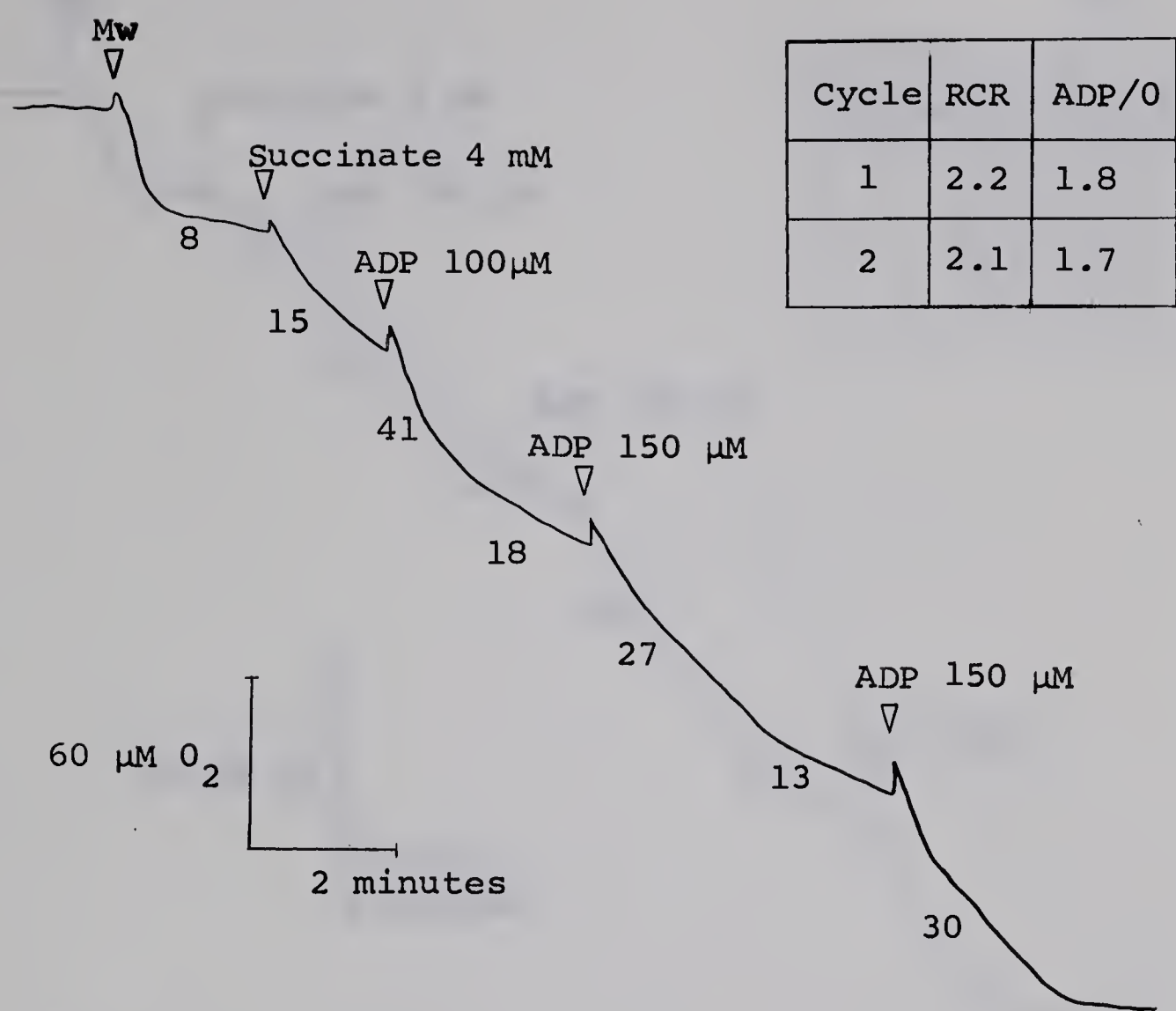


Figure 19. Polarographic trace of the oxygen uptake of mitochondria prepared from one day old bean cotyledons, stored for fifty minutes at 0° C., and assayed in three millilitres of the standard assay medium. 300 micrograms of mitochondrial protein were added, and the assay was carried out at 25° C., using a Gilson Oxygraph. The numbers under the trace indicate the amount of oxygen used in μmoles of oxygen per minute per millilitre of suspension.



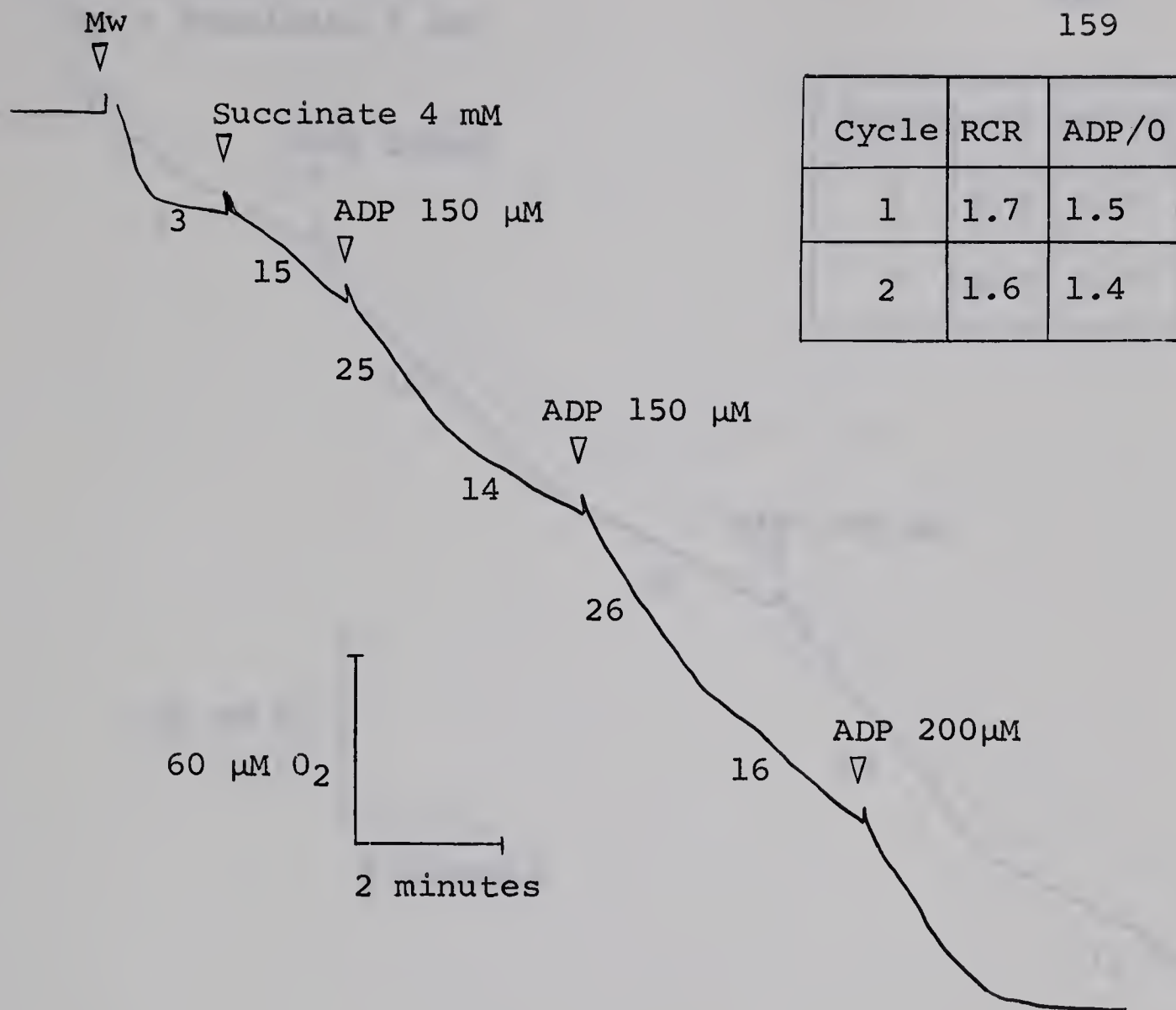


Figure 20. Polarographic trace of the oxygen uptake of mitochondria prepared from one day old bean cotyledons, stored for three hours at  $0^{\circ}\text{C}$ ., and assayed in a final volume of three millilitres of the standard assay medium. 320 micrograms of mitochondrial protein were added per millilitre of the assay medium. Method of assay, and description of the graph symbols is given in figure 19.





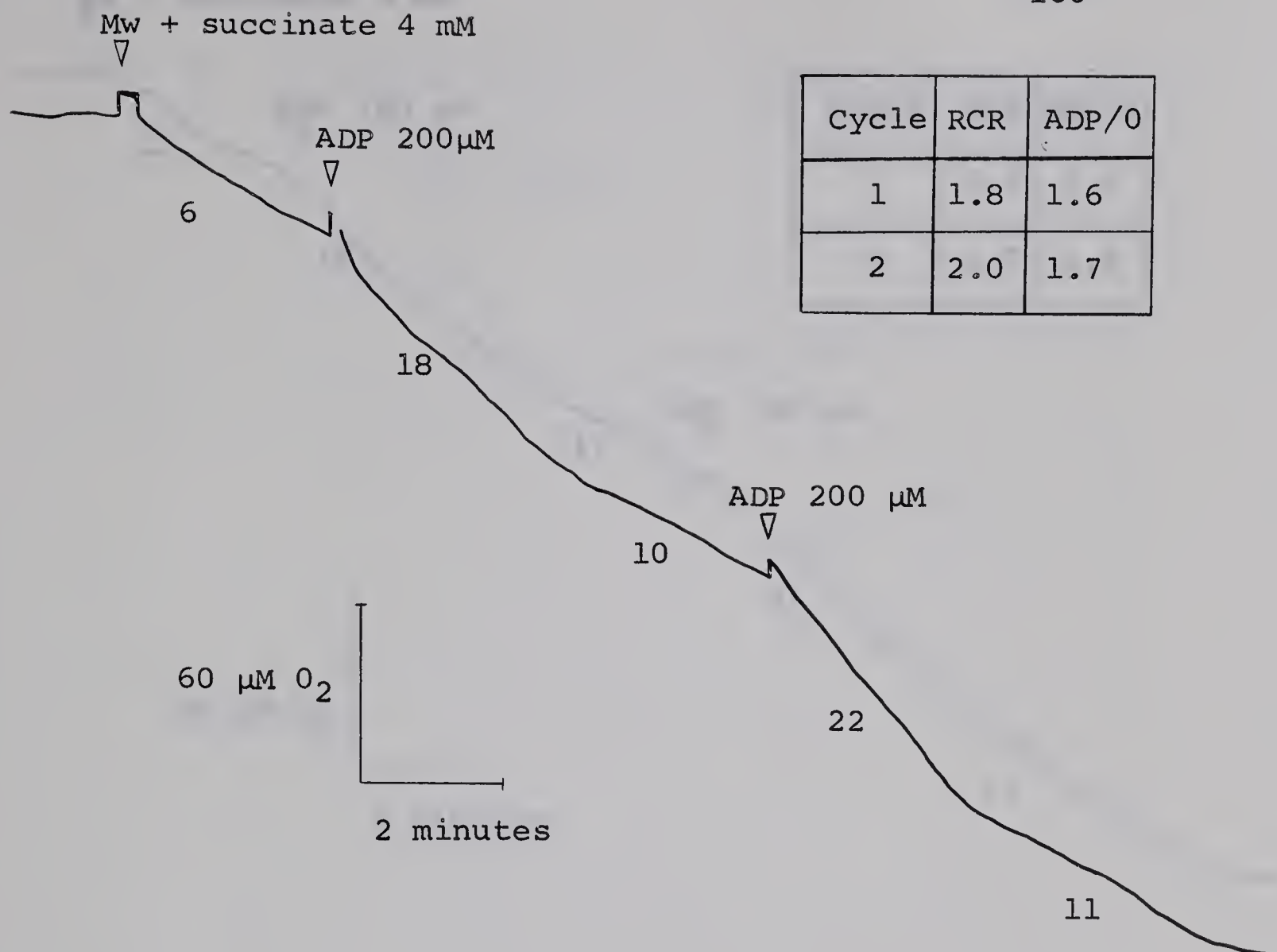


Figure 21. Polarographic trace of the oxygen uptake of mitochondria prepared from three day bean cotyledons, stored for one hour at  $0^{\circ}\text{C}$ ., and assayed in a final volume of three millilitres of the standard assay medium. 300 micrograms of mitochondrial protein were added per millilitre of the assay medium. Method of assay, and description of the graph symbols is given in figure 19.



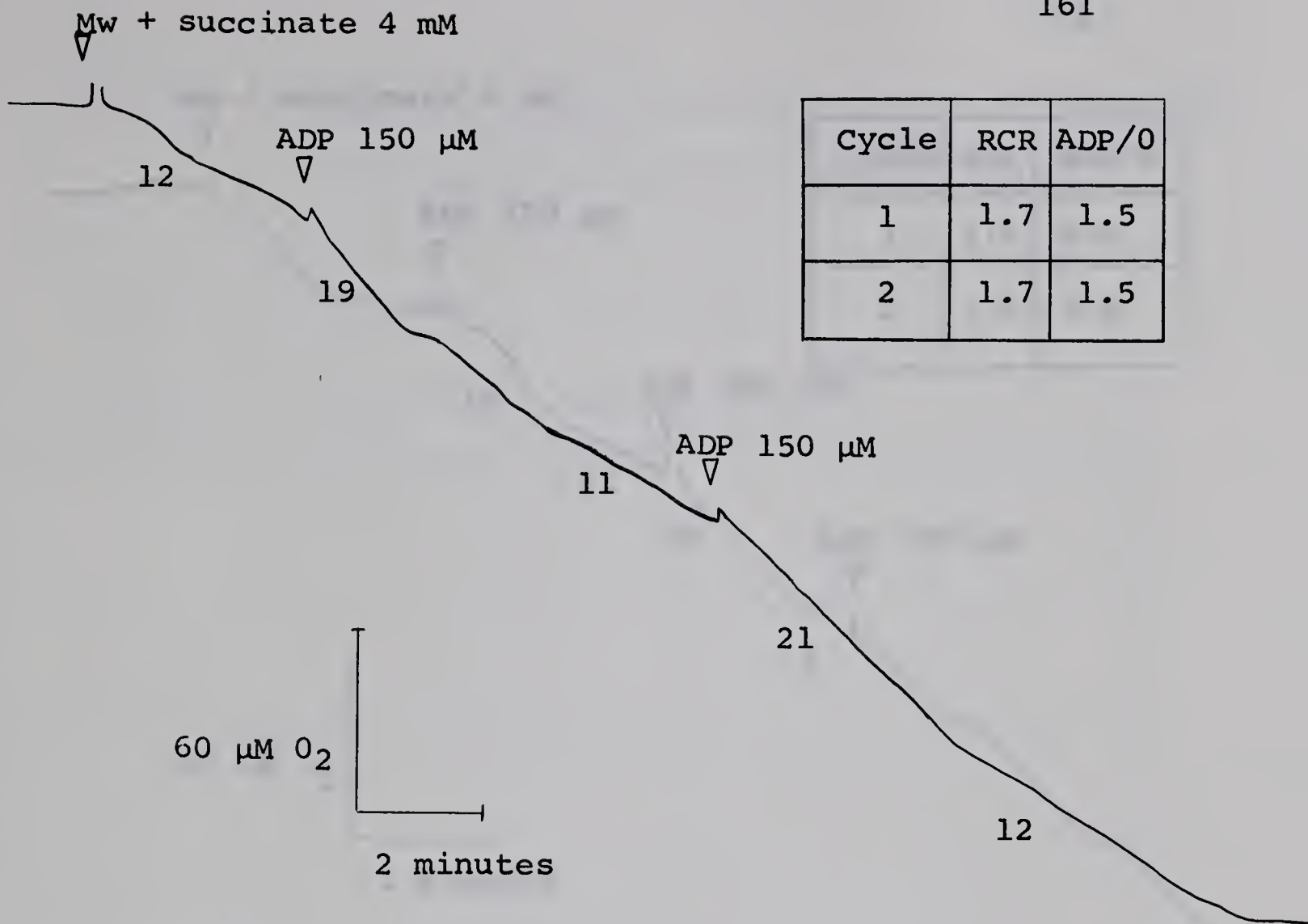


Figure 22. Polarographic trace of the oxygen uptake of mitochondria prepared from three day bean cotyledons, stored for three hours at  $0^{\circ}$  C., and assayed in a final volume of three millilitres of the standard assay medium. 300 micrograms of mitochondrial protein were added per millilitre of the assay medium. Method of assay, and description of the graph symbols is given in figure 19.





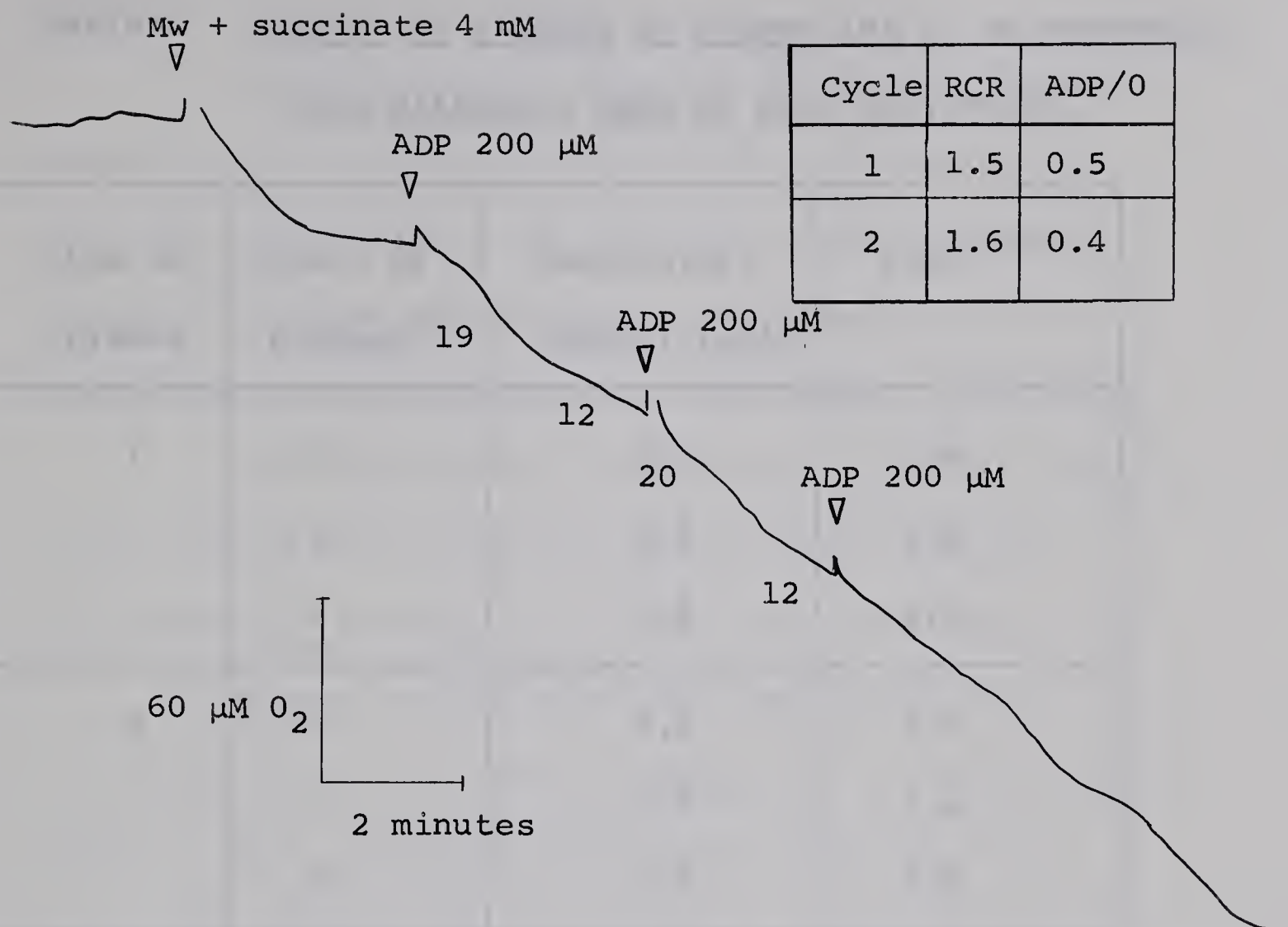


Figure 23. Polarographic trace of the oxygen uptake of mitochondria prepared from six day old bean cotyledons, stored for one hour at 0° C., and assayed in three millilitres (final volume) of the standard assay medium. 400 micrograms of mitochondrial protein were added per millilitre of the assay medium. Method of assay, and description of the graph symbols is given in figure 19.



Table 4. Effect of storage on properties of mitochondria  
from different ages of bean cotyledons\*

Age of tissue	Hours of storage**	Respiratory control ratio***	ADP/O***
1	0	2.5	1.9
	1	2.1	1.8
	3	1.6	1.5
3	0	2.0	1.8
	1	1.9	1.6
	3	1.7	1.5
6	0	1.7	1.7
	1	1.5	0.5
	3	—****	—****

\* Summary of data from figures 10 to 23.

\*\* The bean cotyledon mitochondrial suspension was stored in a test tube in an ice bath.

\*\*\* Average of results from each trace.

\*\*\*\* No figure for oxygen uptake is given, as respiration was very slow, and there was no appreciable response to the addition of substrate or ADP.





## Mitochondrial volume changes

Analysis of the behavior of the mitochondrial suspension in various concentrations of sucrose with both the turbidimetric and electron counting methods indicated a linear relationship between mitochondrial volume and the concentration of the osmotic stabilizer.

The turbidimetric method was preferred because of the continuous record of volume change obtained. The electronic counter was found to be very slow.

Figure 24 indicates the time course of a mitochondrial volume change measured by the turbidimetric method. Bean cotyledon mitochondria suspended in a medium containing 0.50 M sucrose were added to the spectrophotometer cuvettes containing the same medium, but with a lower concentration of sucrose. A fairly rapid swelling was found immediately after the addition of the mitochondrial suspension.

Similar experiments with other concentrations of sucrose are shown in figure 25. An increase in absorbance was found as the sucrose concentration was increased to 0.30 M. When one plots the reciprocal



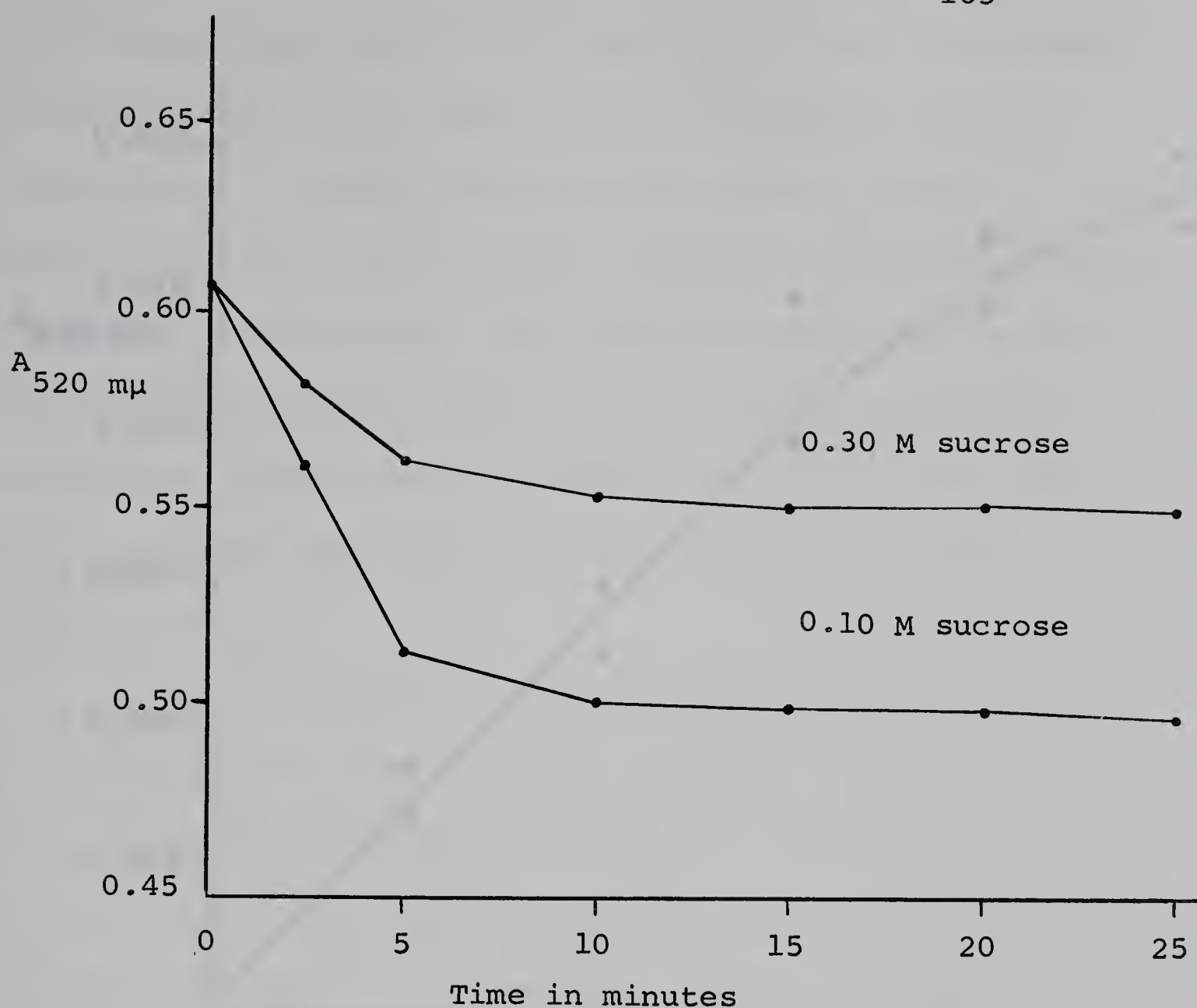


Figure 24. Changes in volume of three day bean cotyledon mitochondria suspended in 0.50 M sucrose, 0.01 M Tris-HCl, and 0.001 M magnesium chloride, pH 7.4 at 25° C, on addition of 0.1 millilitre of mitochondrial suspension (1.0 milligram of mitochondrial protein per millilitre) to 2.9 millilitres of either 0.30 M sucrose, or 0.10 M sucrose, plus 0.01 M Tris-HCl, and 0.001 M magnesium chloride, pH 7.4 at 25° C. Turbidity was measured at 520 m $\mu$ , at 25° C.





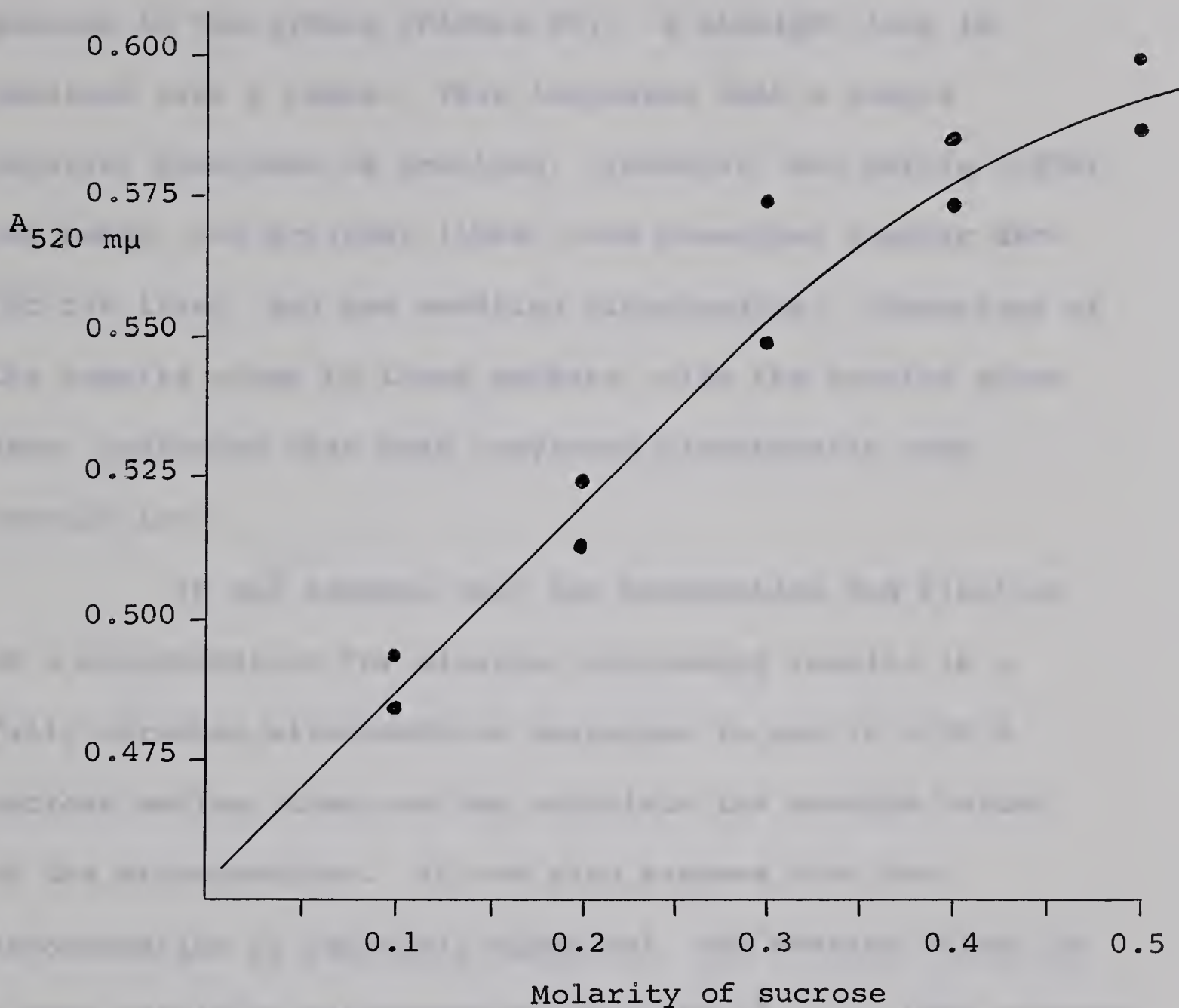


Figure 25. Changes in volume (turbidity) of mitochondrial suspensions as given in figure 24, with changes in the concentration of sucrose in the media. Measurements were made thirty minutes after the addition of the mitochondrial suspension to the medium, to allow completion of the volume change.

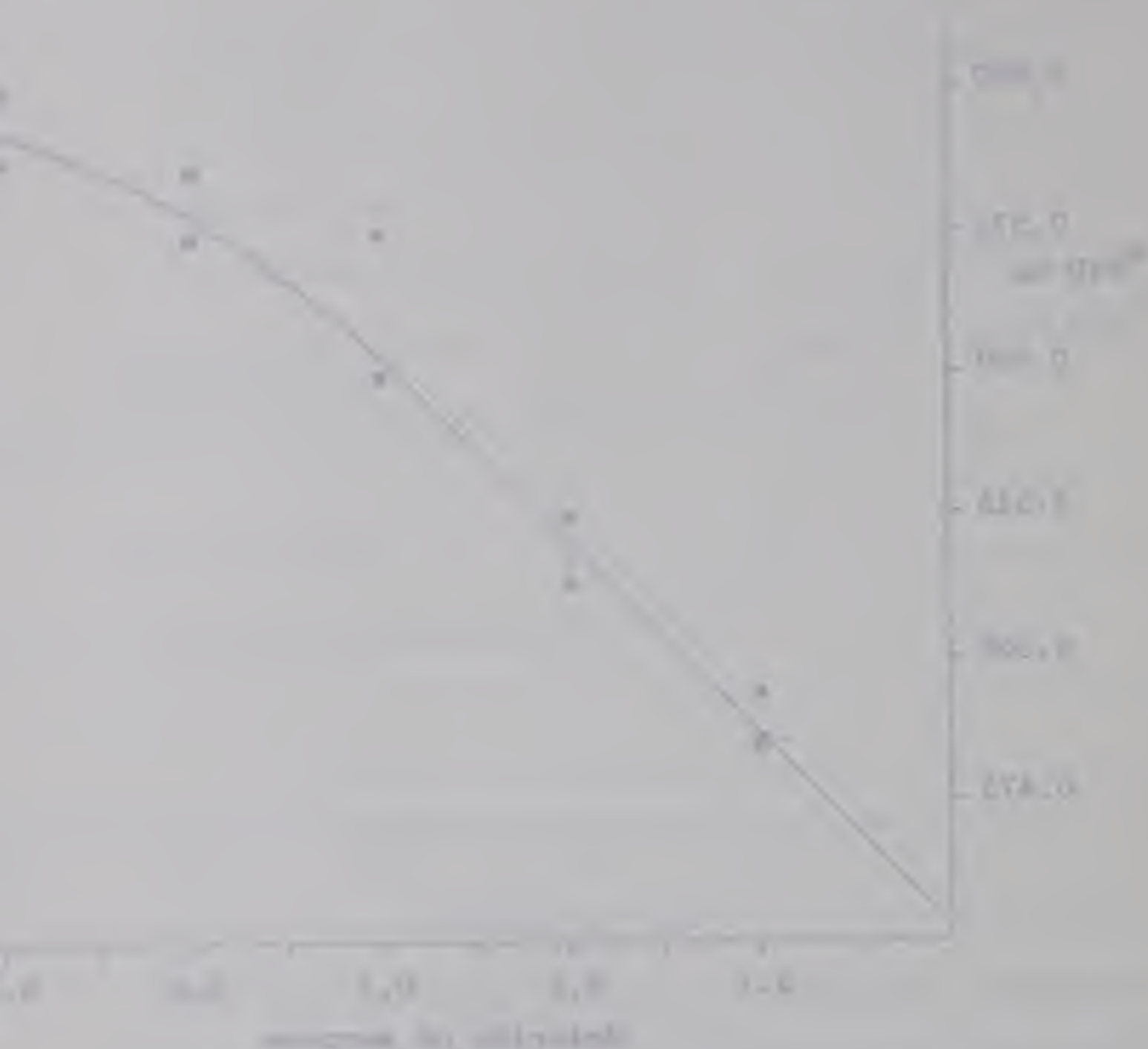


Figure 1. The pH of the solution as a function of the amount of dissolved substance. The data points are shown as open circles, and the line of best fit is shown as a solid line. The pH decreases linearly as the amount of dissolved substance increases.

of the absorbance against the reciprocal of the molarity of sucrose in the system (Figure 26.), a straight line is obtained over a range. This indicates that a simple physical phenomena is involved. Tedeschi, and Harris (1956), and Longo, and Arrigoni (1964) have presented similar data for rat liver, and pea seedling mitochondria. Comparison of the results given by these workers, with the results given here, indicates that bean cotyledon mitochondria obey osmotic law.

If one assumes that the preparation and fixation of a mitochondrion for electron microscopy results in a fully shrunken mitochondrion analagous to one in 0.50 M sucrose medium, then one can calculate the average volume of the mitochondrion. If one also assumes that the mitochondrion is perfectly spherical, the average volume of a bean cotyledon mitochondrion is  $0.150 \mu^3$ . (The average diameter of mitochondria such as those seen in figure 9, was 0.650 microns.) Tedeschi, and Harris (1955) indicate that forty to fifty percent of the mitochondrial volume is osmotically inaccessible. This corresponds fairly closely to the osmotic dead space of the erythrocyte (Lucke, and McCutcheon 1932). Assuming





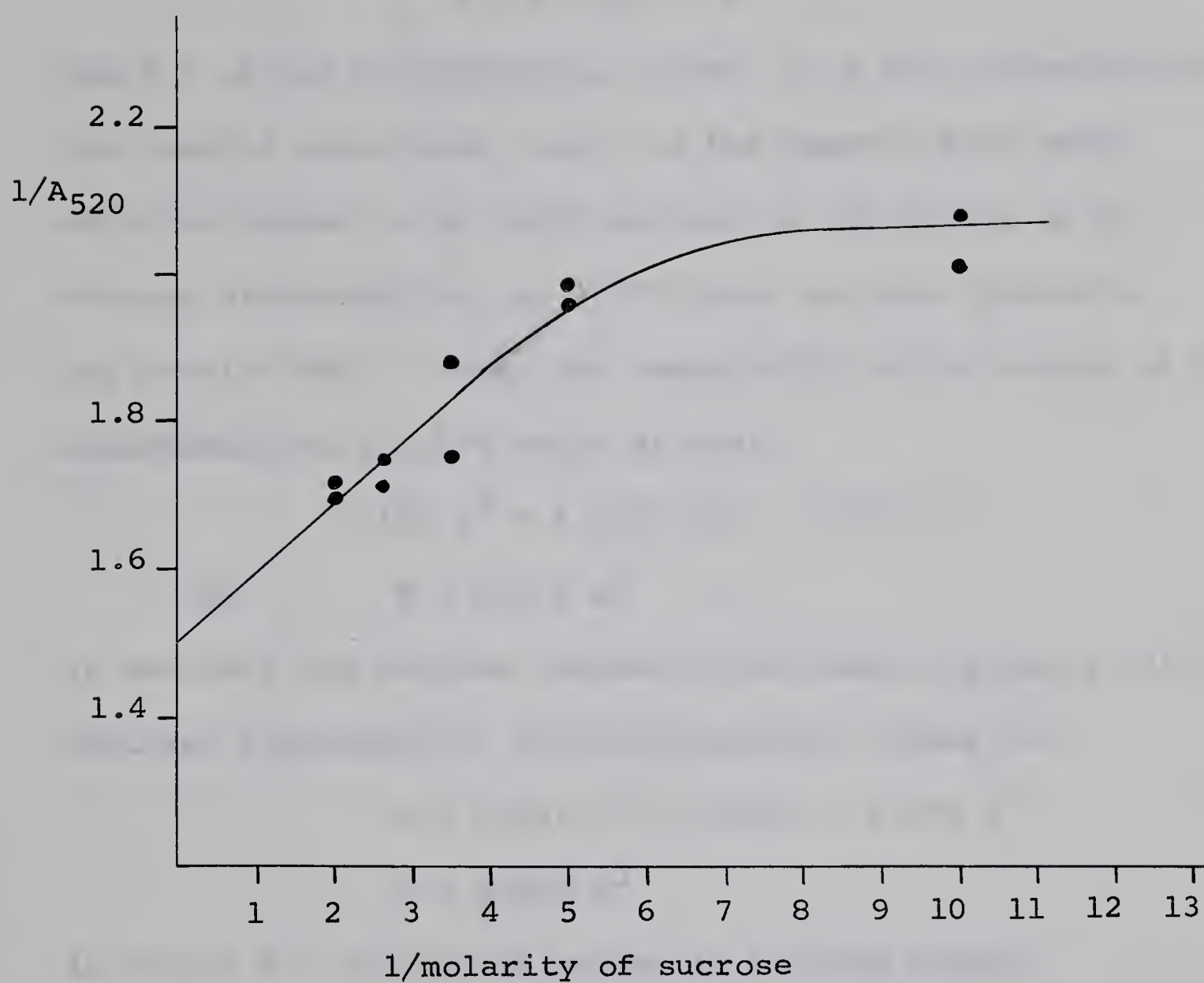


Figure 26. Reciprocal plot of the data obtained in figure 25.



that this is also true for bean cotyledon mitochondria, then one finds that mitochondrial volume increases as:

$$V = K (1/C) + b$$

where  $V$  is the mitochondrial volume,  $C$  is the concentration of the osmotic stabilizer, and  $b$  is the osmotic dead space, which we assume to be fifty percent of the volume of an average mitochondrion, or 0.075 cubic microns (Tedeschi, and Harris 1955). Thus, the osmotically active volume of the mitochondrion is 0.075 cubic microns.

$$0.150 \mu^3 = K (1/0.50) + 0.075 \mu^3$$

$$\text{or} \quad K = 0.037 \mu^3$$

At one-half the sucrose concentration used to produce fully shrunken mitochondria, the mitochondrial volume is:

$$V = 0.037 \mu^3 (1/0.25) + 0.075 \mu^3$$

$$V = 0.225 \mu^3$$

In figure 27, calculated volume is plotted against sucrose concentration

As the electronic sizing of mitochondria was a very slow process (approximately one-half minute per reading), this procedure was not used extensively. Even after five filterings of the medium used in the analysis, there was a very high blank count at the lower end of





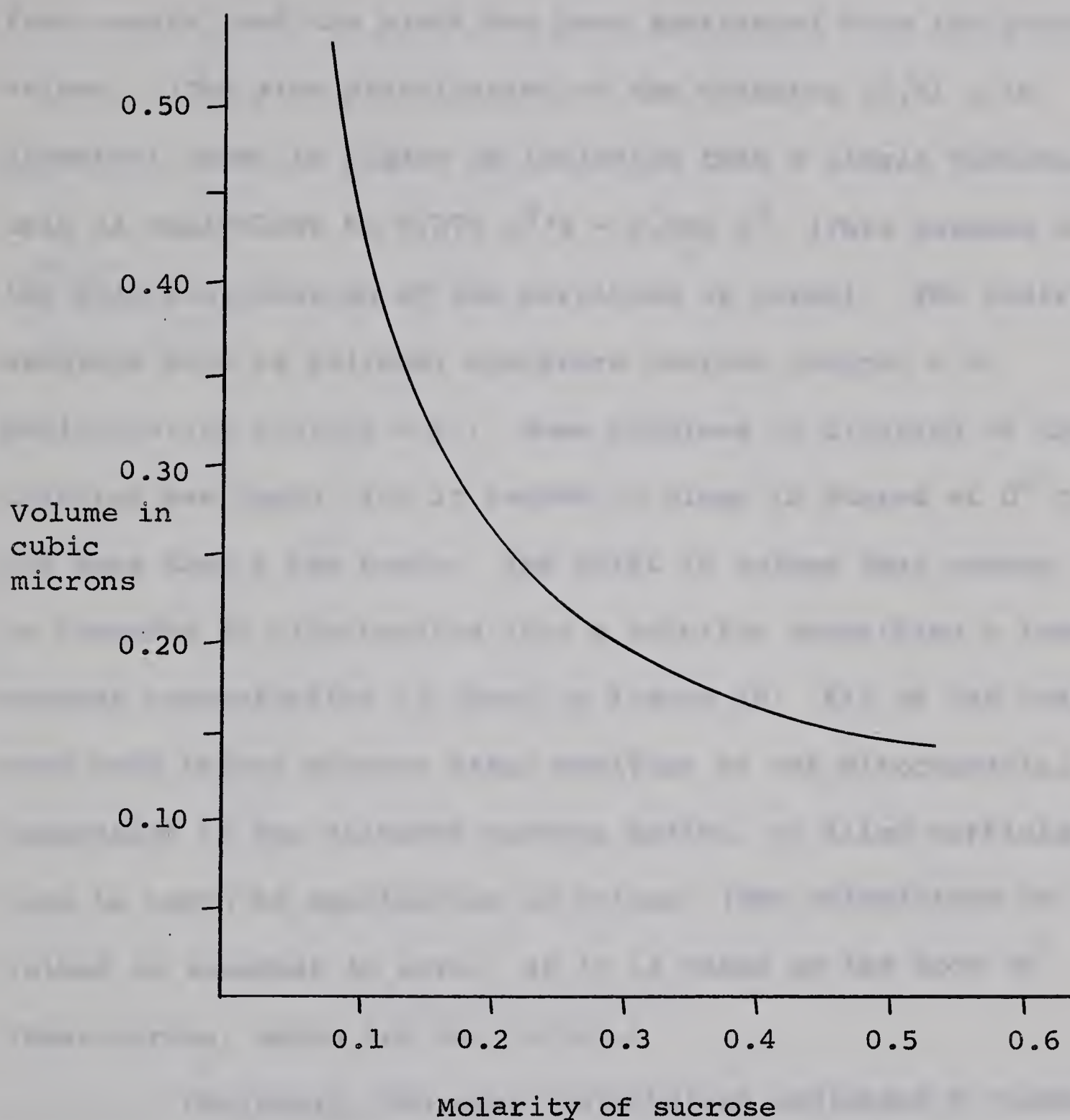


Figure 27. Plot of the calculated volume of a mitochondrion versus the sucrose concentration of the medium in which the mitochondrion is suspended.



the size scale. The results given are the average of at least four counts, and the blank has been subtracted from the plotted values. The size distribution of the Colabtex ( $0.81 \mu$  in diameter) shown in figure 28 indicates that a single threshold unit is equivalent to  $0.276 \mu^3/4 = 0.069 \mu^3$ . (This assumes that the size distribution of the particles is normal. The instrument settings were as follows; Aperature current control = 8, Amplification control = 8.) Some problems in dilution of the Colabtex was found, for it tended to clump if stored at  $0^\circ \text{C}$ . for more than a few hours. The shift in volume that occurs on transfer of mitochondria into a solution containing a lower sucrose concentration is shown in figure 29. All of the tests were made thirty minutes after addition of the mitochondrial suspension to the filtered sucrose medium, to allow sufficient time to reach an equilibrium in volume. (The calculation of volume is somewhat in error, as it is based on the mode of these curves, which are not normal.)

Obviously, the size distribution indicates a volume three to four times that calculated from electron micrographs. This could be explained by coincidence of entry of several mitochondria through the pore, or by a general clumping of the the mitochondria during the incubation period.

The volume of a mitochondrion was calculated





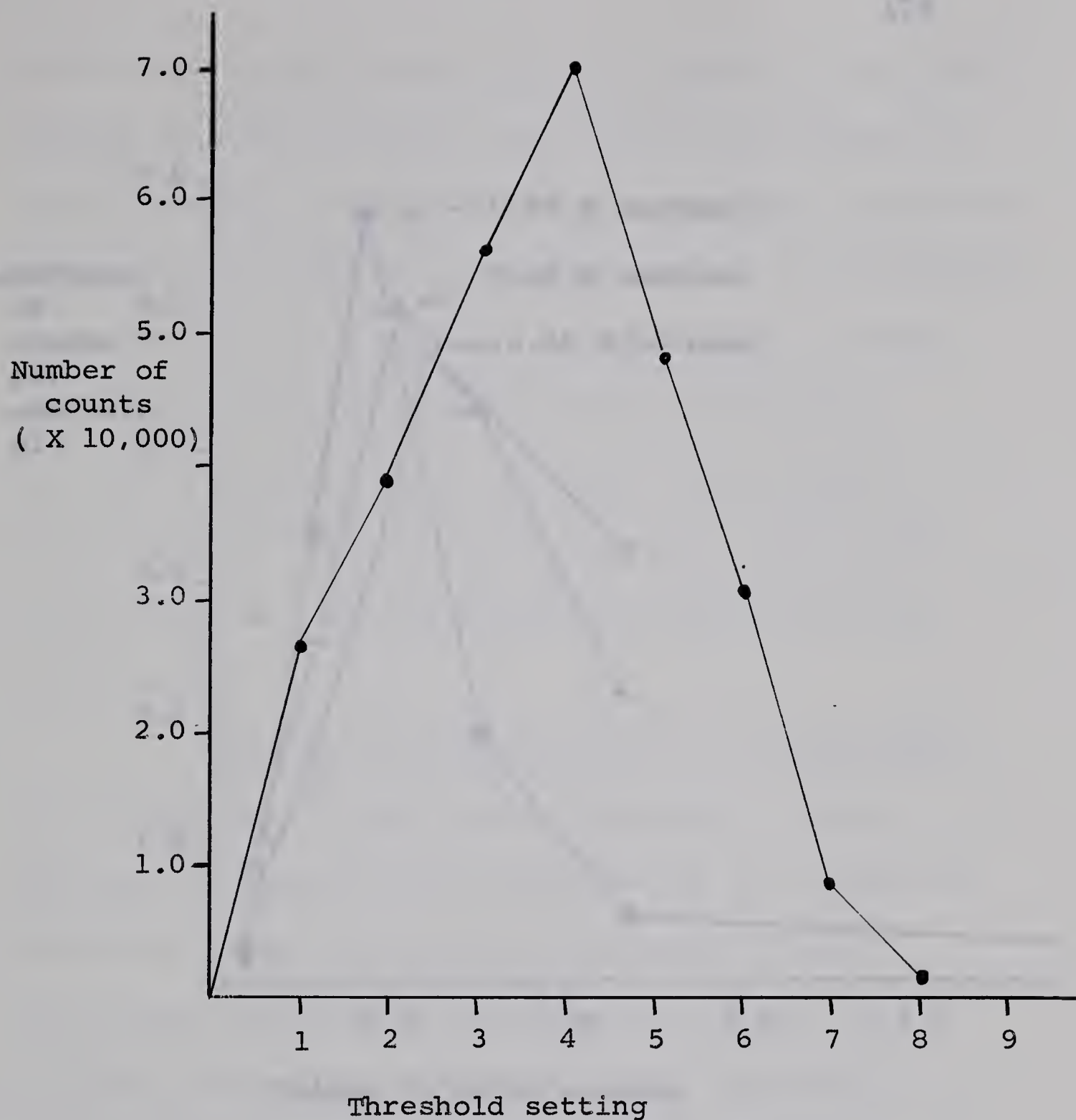


Figure 28. Measurement of the volume of latex particles suspended in 0.30 M sucrose, 0.01 M Tris-HCl, and 0.001 M magnesium chloride, using a Coulter counter (model B) with a 30  $\mu$  pore. The analysis was done at 25° C.



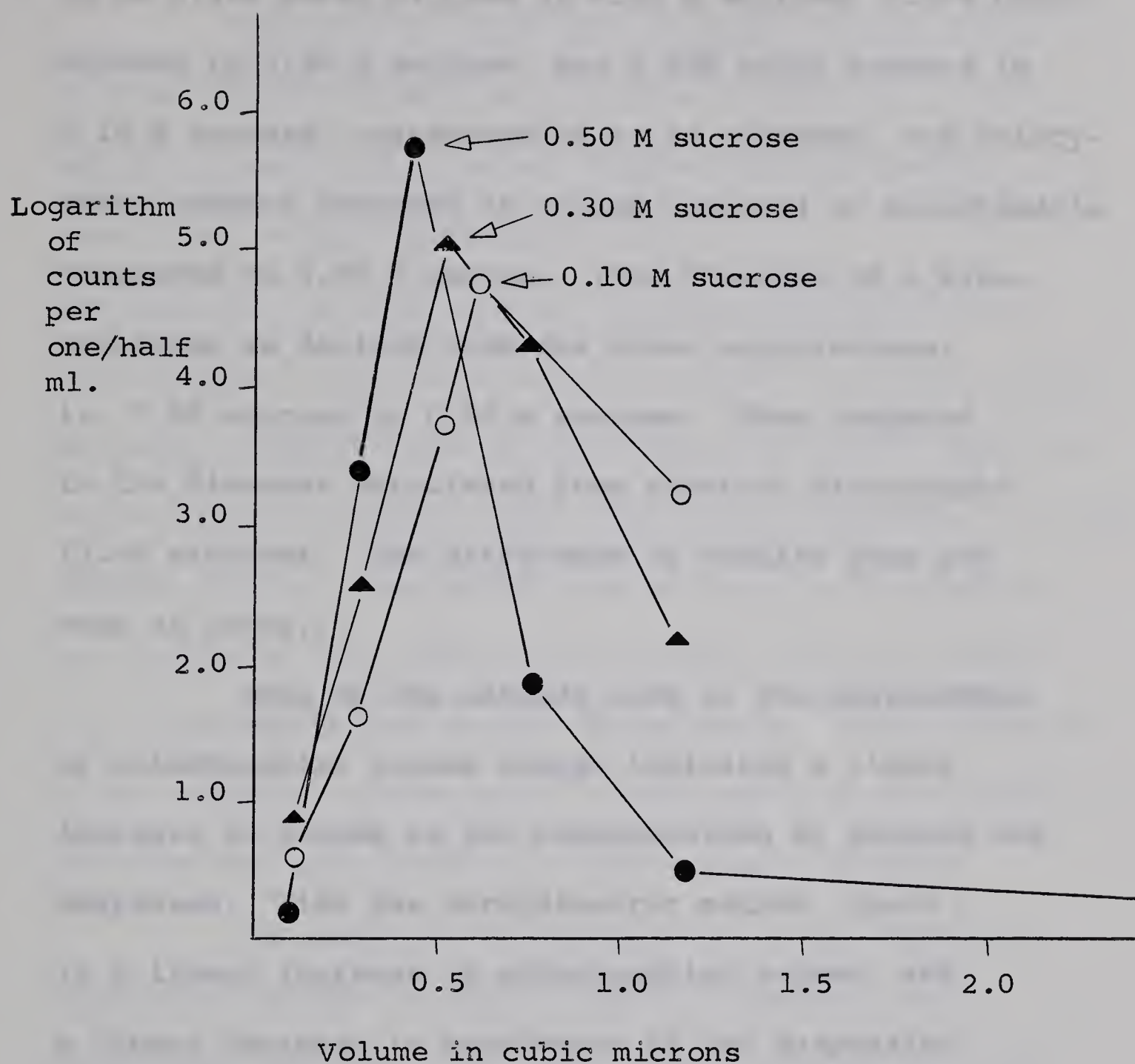


Figure 29. Measurement of mitochondrial volume with Coulter Counter Model B. 0.1 ml. of a three day bean mitochondrial suspension in the standard assay medium was added to 10.0 ml. of the same medium containing the concentrations of sucrose given on the graph, allowed to equilibrate for 30 minutes at 25° C., and then measured in a thermostatted cuvette at 25° C.

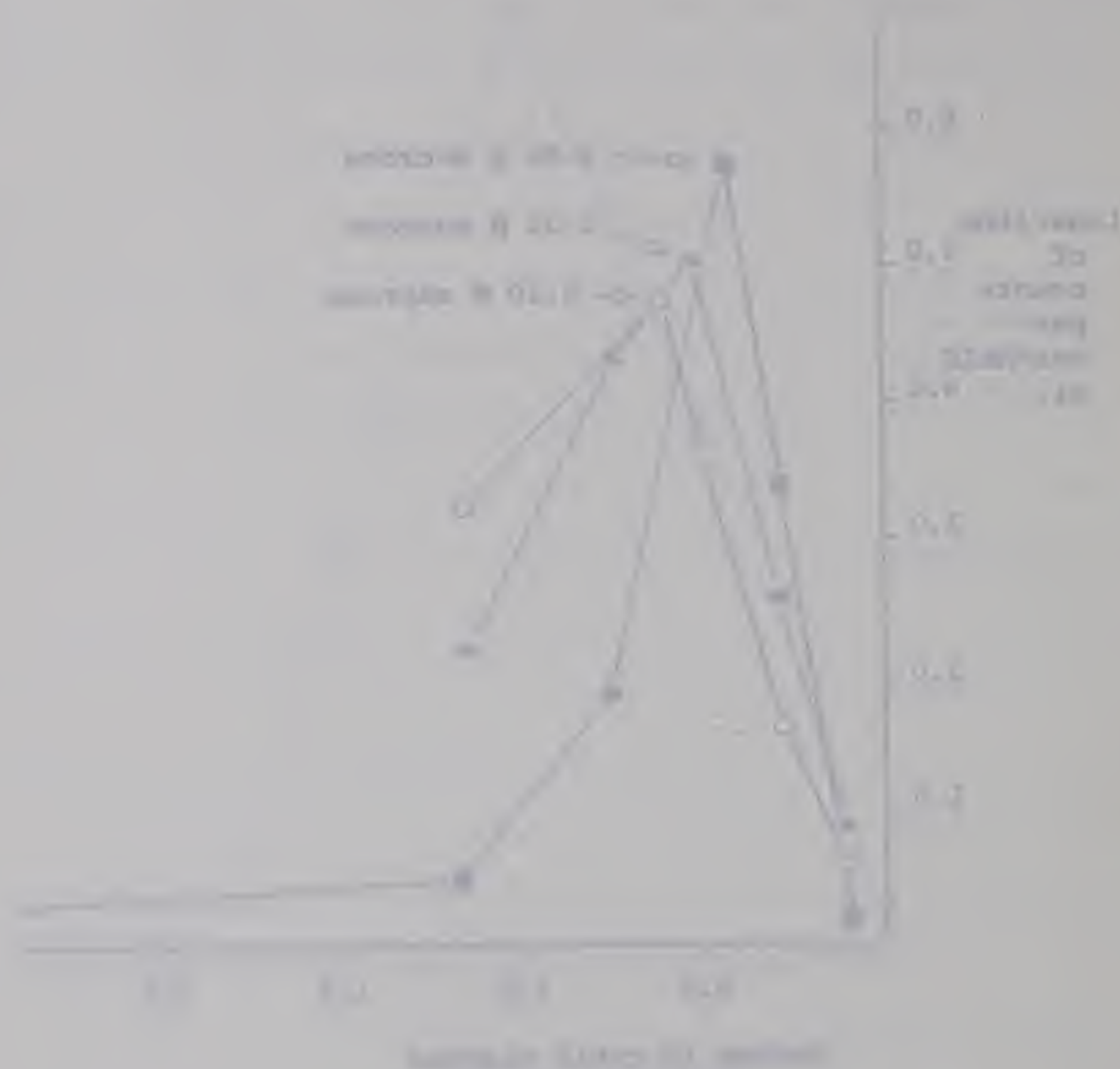


Figure 1. Power (1 -  $\beta$ ) versus sample size (n) for different levels of significance ( $\alpha$ ). The curves show that power increases with sample size and decreases with the level of significance. The power is highest for  $\alpha = 0.05$  and lowest for  $\alpha = 0.10$ .

The power of a statistical test is the probability of rejecting the null hypothesis when it is false. The power of a test is a function of the sample size, the level of significance, and the effect size. The power of a test is highest when the sample size is large, the level of significance is high, and the effect size is large.



to be 0.425 cubic microns in 0.50 M sucrose, 0.504 cubic microns in 0.30 M sucrose, and 0.588 cubic microns in 0.10 M sucrose, corresponding to an eighteen, and thirty-seven percent increase in volume compared to mitochondria suspended in 0.50 M sucrose. (The diameter of a mitochondrion as derived from the above calculations, is 0.93 microns in 0.50 M sucrose. When compared to the diameter calculated from electron micrographs (0.65 microns), the difference in results does not seem as great.)

Both of the methods used in the measurement of mitochondrial volume change indicated a linear increase in volume as the concentration of sucrose was decreased. With the turbidimetric method, there is a linear increase in mitochondrial volume, and a linear decrease in absorbance of the suspension as the sucrose concentration in the suspending medium is decreased. Thus, one would expect turbidity measurements to adequately describe mitochondrial volume changes. (No comparable analysis of the effect of osmotic concentration on rat liver or yeast mitochondrial volume was done. Results given in the sections on rat liver, and yeast mitochondria indicated that these organelles behaved in a normal manner.)



Mitochondria prepared from the various ages of bean cotyledons studied responded to swelling, and shrinking agents in a manner similar to mitochondria from other sources (Lehninger 1964).

The addition of 5.0 millimolar calcium chloride to a mitochondrial suspension caused a gradual swelling (Figure 30.). Initially, the addition of ATP (curve A) caused a decrease in the rate of swelling, but very little shrinkage. Further studies (curve B) indicated that this lack of response was caused by late addition of the ATP, that is, after the mitochondria had swollen excessively. Total reversal to the original volume prior to the addition of calcium chloride was only shown in a small number of runs.

The addition of phosphate to a mitochondrial suspension caused a similar type of swelling (Figure 31.), except that the volume change could be reversed by ATP even after the mitochondria had reached an equilibrium at a larger volume. This would indicate that either phosphate induces a different type of swelling than calcium, or that both cause swelling by the same mechanism, but calcium causes uncoupling when the mitochondria attain a new equilibrium in volume. Lehninger





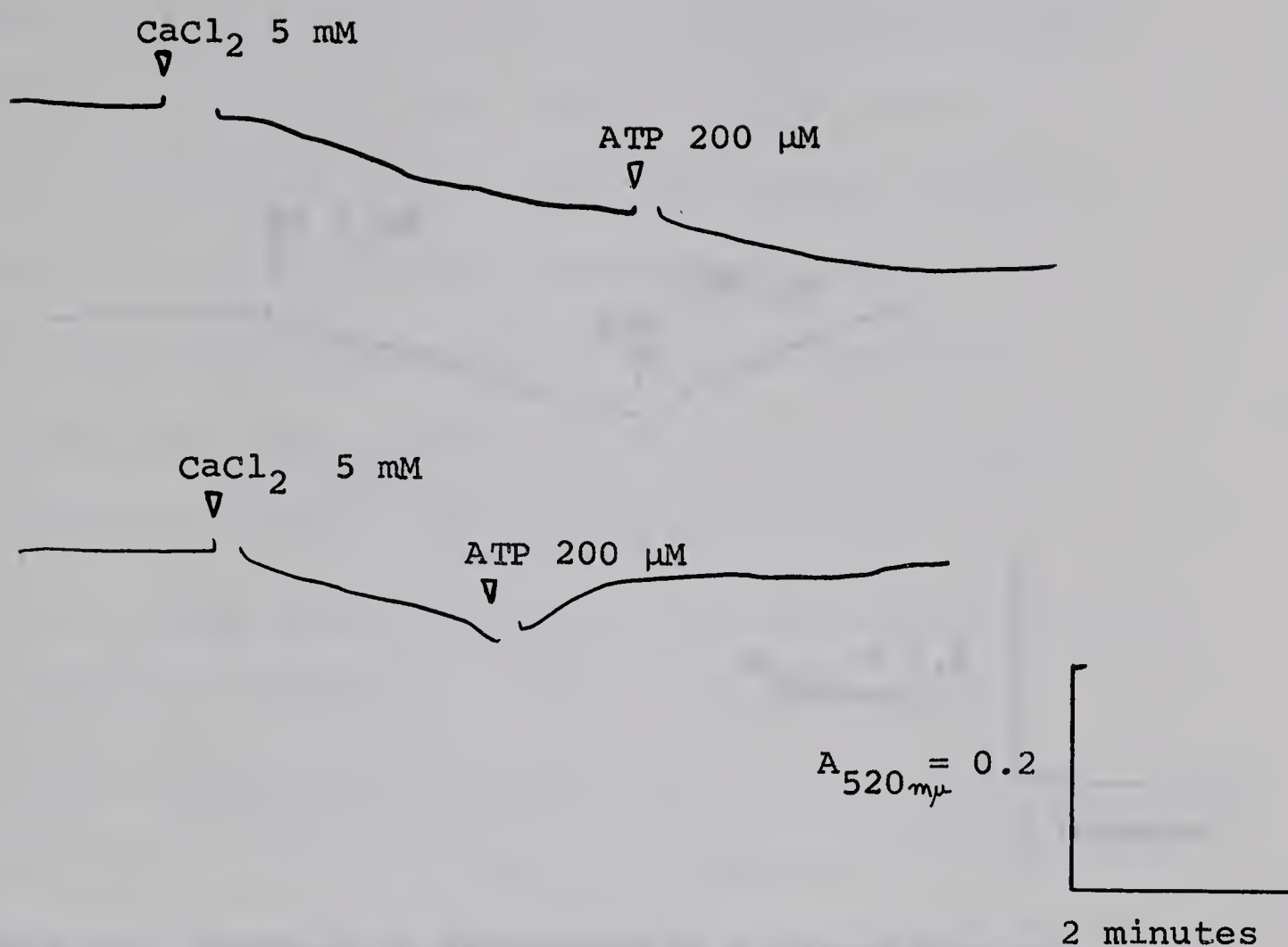


Figure 30. Changes in absorbance of a suspension of mitochondria prepared from three day old bean cotyledons (400 micrograms of mitochondrial protein per millilitre) on addition of calcium chloride, and ATP. The standard assay medium was used, and the test was carried out at 25° C.



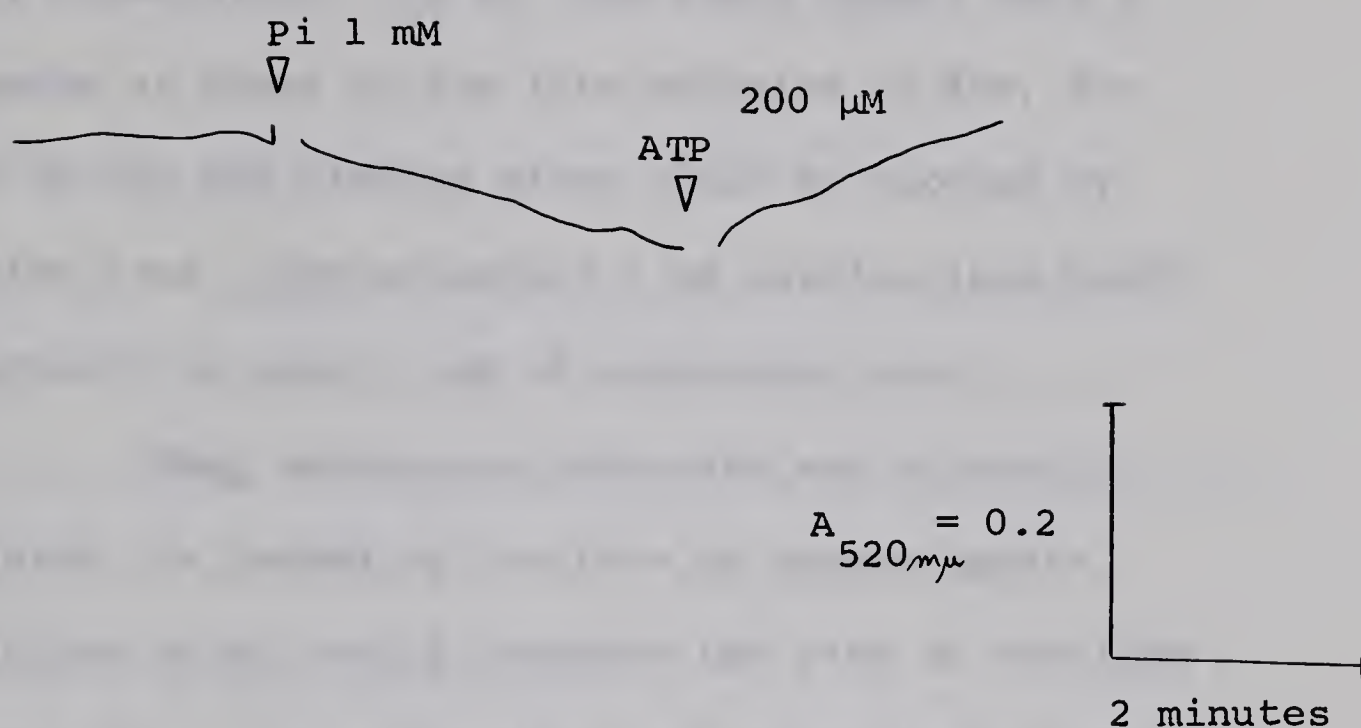


Figure 31. Changes in absorbance of a suspension of mitochondria prepared from three day bean cotyledon mitochondria (400 micrograms of mitochondrial protein per millilitre) on addition of phosphate (Pi), and ATP. The standard assay medium was used, and the assay was carried out at 25° C.





(1962) has suggested that calcium acts by competing with magnesium ions for the role of co-ordinating ATP, or ADP with the mitochondrial enzymes responsible for their conversions. If so, one would expect such a response as shown on the late addition of ATP, for most of the ATP binding sites would be blocked by calcium ions. (Approximately 5 mM calcium ions would be present to about 1 mM of magnesium ions.)

When endogenous substrate was virtually depleted, as judged by the rate of oxygen uptake, additions of ADP would increase the rate of swelling (Figure 32.). If substrates, or phosphate had been added previously, shrinkage occurred when ADP was added (Figure 33.). This indicates that phosphorylation of ADP must be in progress if the mitochondria are to decrease their volume.

In figure 34, a gradual swelling occurring after the addition of phosphate is shown. If one considers the concentration of ADP to be one of the controlling factors in mitochondrial swelling, a gradual swelling should occur when little respiration is evident, as the ADP concentration increases. On the addition of a substrate, the ADP concentration will decrease as



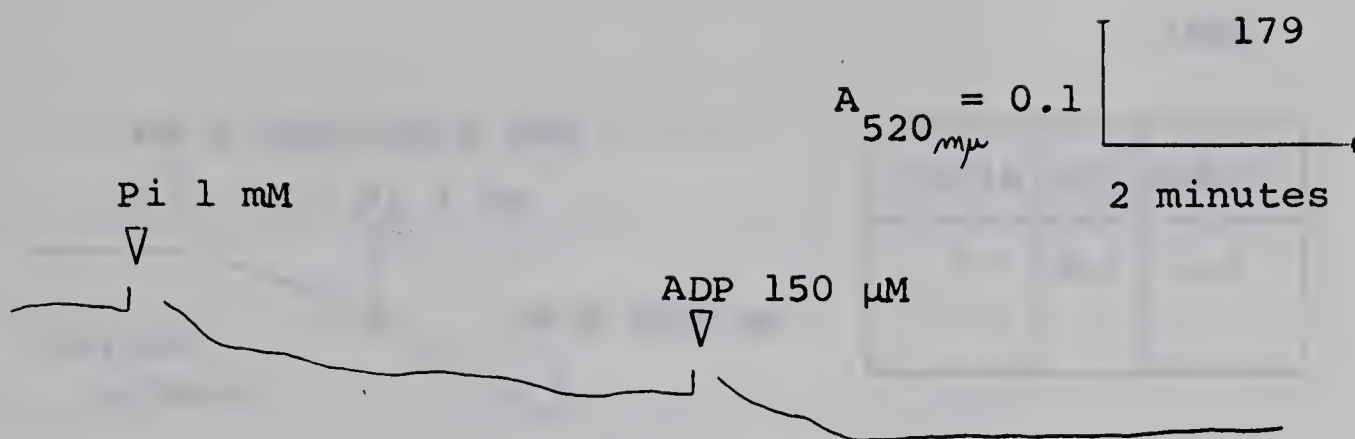


Figure 32. Changes in absorbance of a mitochondrial suspension prepared from three day bean cotyledon (350 micrograms of mitochondrial protein per millilitre) on addition of phosphate (Pi), and ADP, without the addition of a substrate.

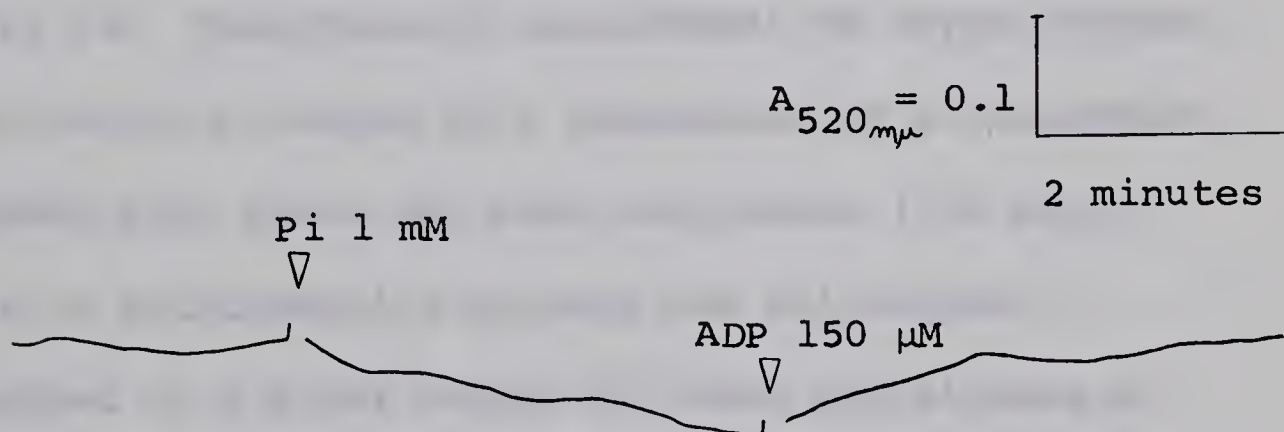


Figure 33. Changes in absorbance of a mitochondrial suspension prepared from three day bean cotyledons (350 micrograms of protein per millilitre) on addition of phosphate (Pi), and ADP to the suspension to which 4 mM succinate had previously been added.





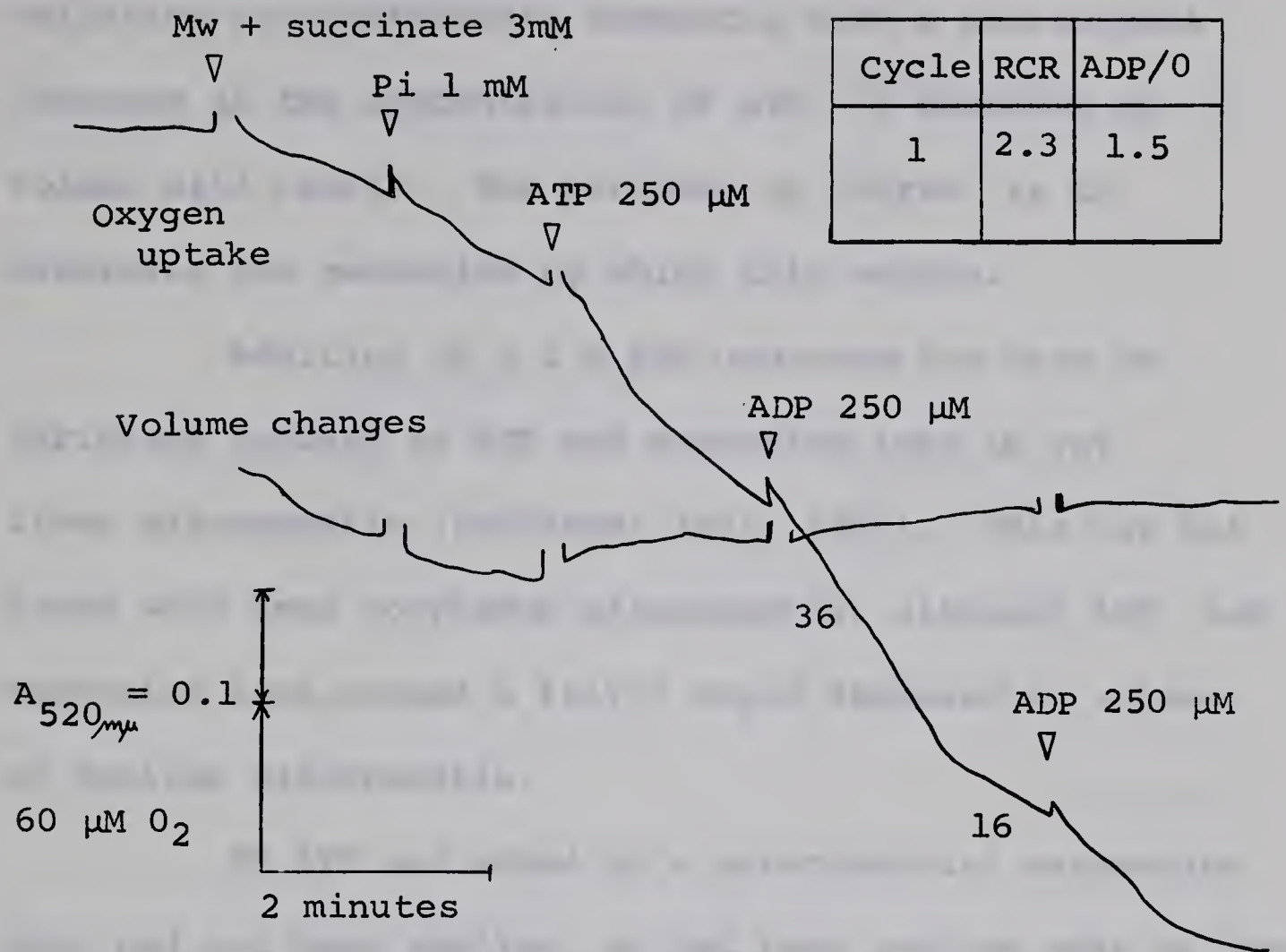


Figure 34. Simultaneous measurement of oxygen uptake and absorbance changes of a suspension of mitochondria prepared from three day bean cotyledons (300 micrograms of mitochondrial protein per millilitre) suspended in a final volume of three millilitres of the standard assay medium. The test was carried out at 25° C., using the polarographic unit constructed for use in the spectrophotometer.



oxidative phosphorylation commences with a concomitant increase in the concentration of ATP. A decrease in volume will result. The problem, of course, is to determine the mechanism by which this occurs.

Addition of 0.1 % BSA increases the rate of shrinkage induced by ATP and magnesium ions in rat liver mitochondria (Lehninger 1962, 1964). This was not found with bean cotyledon mitochondria, although ATP, and magnesium ions caused a fairly rapid decrease in volume of swollen mitochondria.

If ATP was added to a mitochondrial suspension that had not been swollen, or had been swollen only by the addition of substrate, only a small amount of shrinkage was found, thus indicating that the medium was possibly hypertonic to some extent (Figure 35.).

In a number of mitochondrial preparations, ATP was shown to cause a decrease in the rate of oxygen uptake for periods of up to thirty seconds (Figure 36.). This indicates that ATP can control respiration as well as induce a decrease in mitochondrial volume. The same mechanism may be involved in both effects, for by causing the mitochondrial shrinkage, ATP will also limit the entry of substrates. Here lies another problem, for





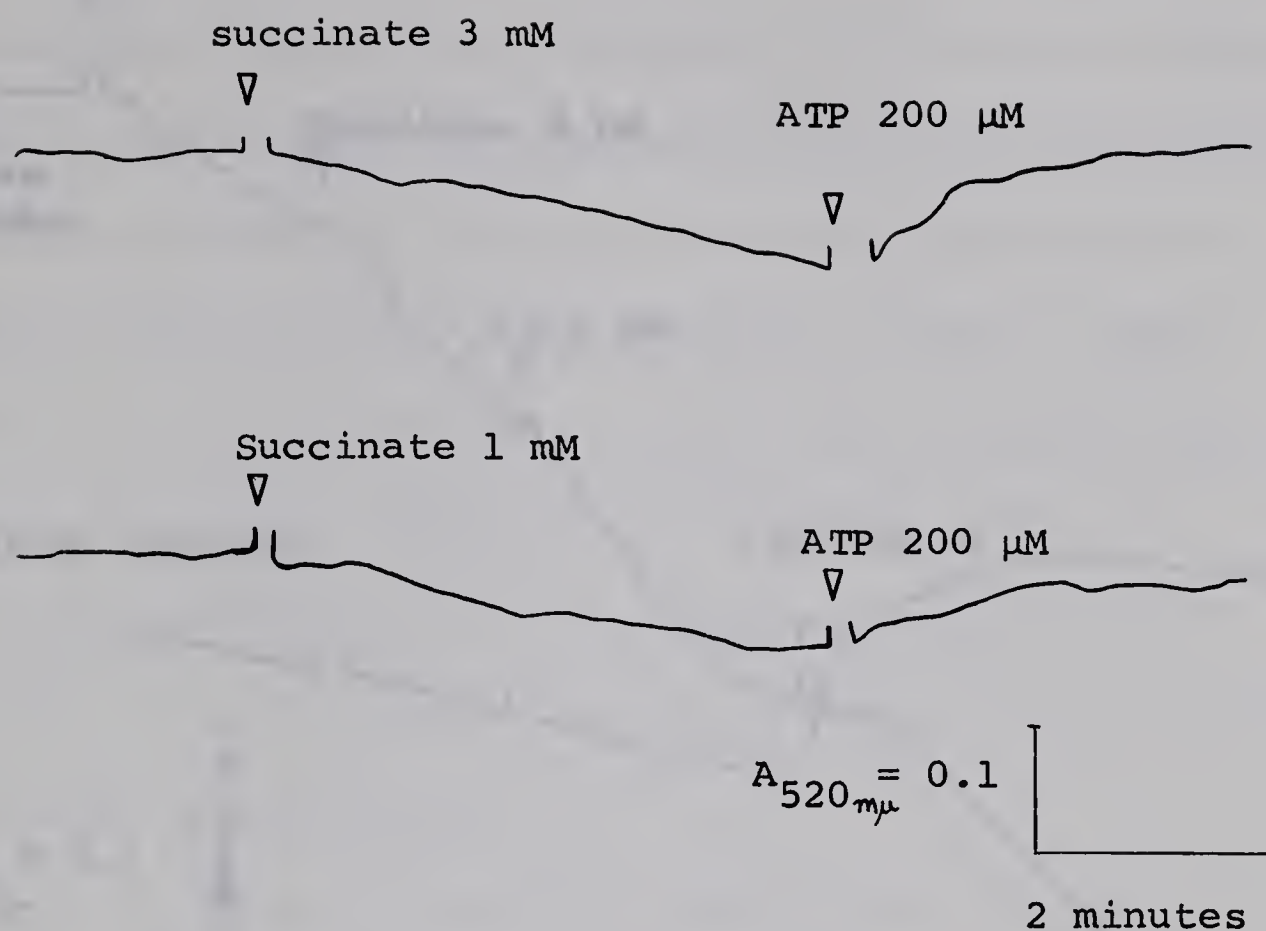


Figure 35. Changes in absorbance of a mitochondrial suspension prepared from three day bean cotyledons (350 micrograms of protein per millilitre), which have been allowed to gradually swell on the addition of substrate, and then treated with ATP.



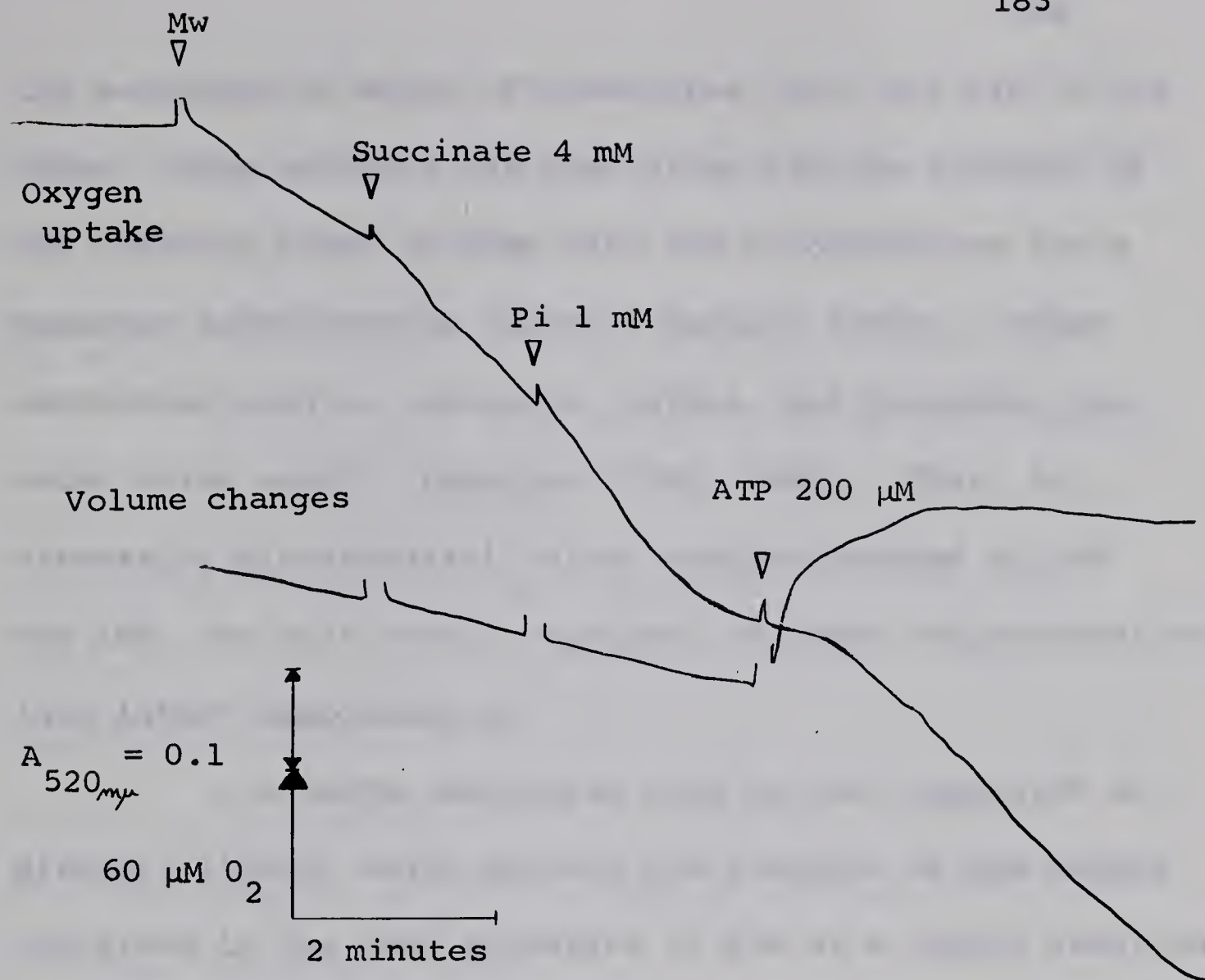


Figure 36. Simultaneous measurement of oxygen uptake and absorbance changes of a suspension of mitochondria prepared from three day bean cotyledons (350 micrograms of mitochondrial protein per millilitre) suspended in a final volume of three millilitres of the standard assay medium. The test was carried out at 25° C., using the polarographic unit constructed for use in the spectrophotometer.





the mechanism of entry of substrates, ADP, and ATP is not known. Some evidence has been given for the transfer of the reducing power of NADH into the mitochondrion via a membrane dehydrogenase (Cereijo-Santalo 1966a). Other substrates such as succinate, malate, and phosphate can enter quite easily (Lehninger 1962, 1964). Thus, in discussing mitochondrial volume changes induced by ATP and ADP, one must remain cognizant of their non-penetration into intact mitochondria.

A transfer mechanism such as that suggested by Mitchell (1966b) would explain the transfer of the energy contained in the bond structure of ATP as a cyclic reaction with a phosphate being transferred from the external ATP to an internal ADP, forming an internal ATP. This assumes that the energy inherent in ATP must be transferred completely through the membrane, and it is possible that it need not be. When one combines the theory of Mitchell (1966b) with that of Cereijo-Sanatalo (1966a), that is, preferential movement of ions into the membrane, and changes in volume of the protein gel present in the matrix, one can see that ATP, or the energy inherent in its bonds need not pass completely through the mitochondrial membrane.



No differences among ages of tissue were found with regards to mitochondrial volume changes. This result may reflect insufficiently sensitive equipment.

The effect of ethylene on bean cotyledon mitochondria

Mitochondrial preparations treated by incubation in the standard assay medium, containing either 100 parts per million of ethylene, or completely saturated with ethylene did not show an appreciable change in oxygen uptake, respiratory control, or phosphorylation of ADP on addition of ADP to the Oxygraph cuvette, even after incubation for thirty minutes at 25° C. Similar results were shown by Lyons, and Pratt (1964).

When a portion of a mitochondrial suspension was added to solutions containing from 100 parts per million to saturation, no change in volume compared to untreated suspensions was found, except when the medium was saturated with ethylene. Since essentially anaerobic conditions were present, respiration dependent volume changes could not occur under the latter conditions.

To measure a possible difference in the rate of volume change in ethylene treated, and untreated mitochondria, the spectrophotometer was used as a differential unit. Ethylene was found to increase the



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rate of shrinkage induced by ATP, or ADP with substrate, over the rate of shrinkage induced by the same agents in the sample not treated with ethylene (Figure 37.). When greater than five hundred parts per million of ethylene was used as the treatment, the differences in the rates of volume change decreased sharply.

Although ethylene treatment increased the rate of swelling induced by ADP in mitochondrial preparations exhausted of substrate, it did not increase the rate of swelling caused by calcium, or phosphate. Lyons, and Pratt (1964) indicated that ethylene slightly increased the rate of mitochondrial swelling caused by calcium, or phosphate, but only in potassium chloride media. In a series of experiments with potassium chloride, sucrose, or mannitol media, we showed that there was little difference in the response to ethylene treatment in any of these media. No change in the rate of mitochondrial swelling induced by phosphate, or calcium, after ethylene treatment, was noted.

From calculations based on the turbidity changes observed in the presence of ethylene, the gas, at the concentrations used, could account for significant mitochondrial respiration changes. The change in turbidity of a mitochondrial suspension induced by ADP or ATP after treatment with ethylene can be related to the actual



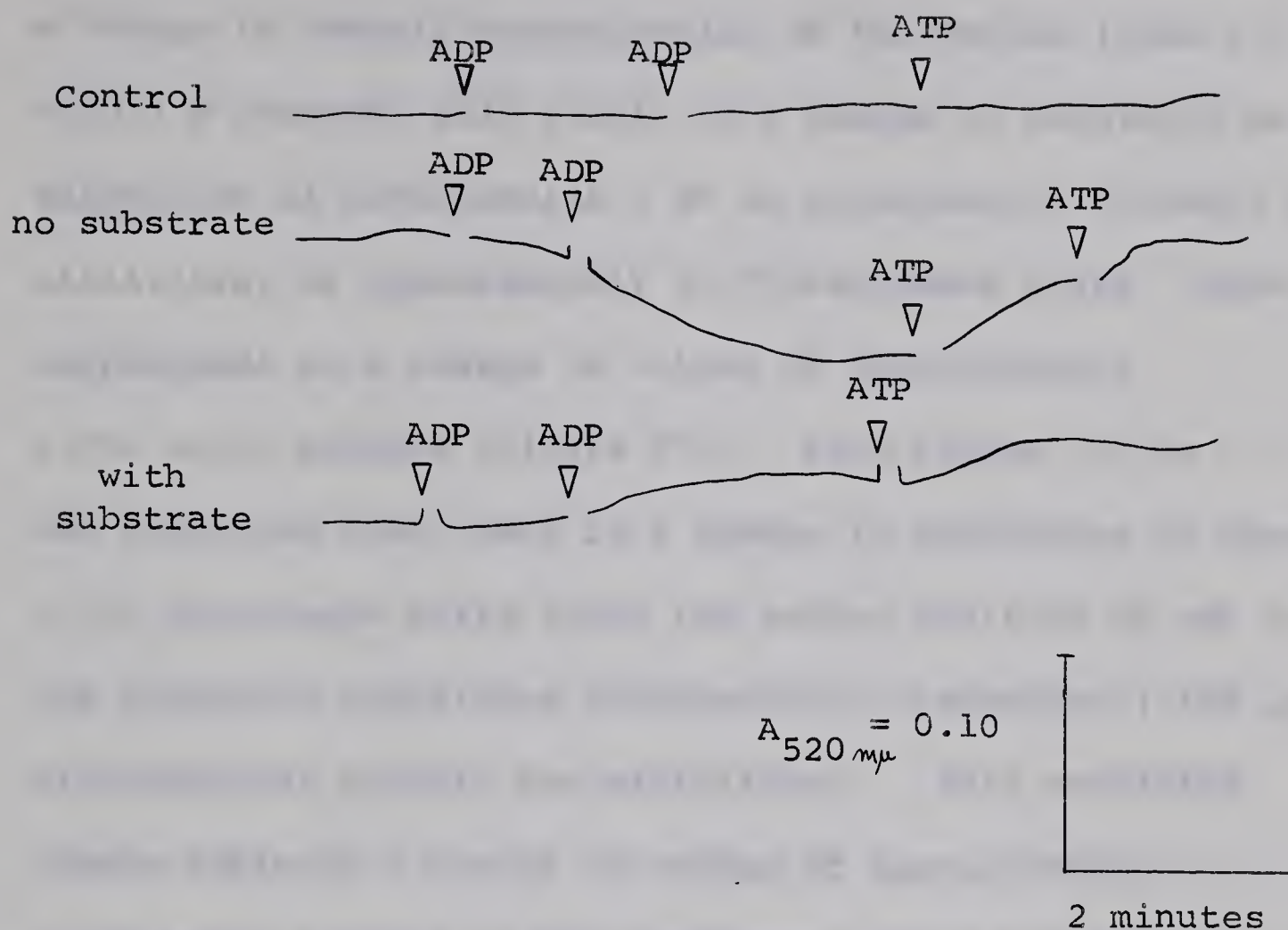


Figure 37. Differential spectrophotometry of suspensions of mitochondria prepared from three day bean cotyledons. (400 micrograms of protein were added to each cuvette) One cuvette contained 100 parts per million of ethylene added as a solution in the standard assay medium used in the test. Succinate was the substrate used, and was added prior to the addition of the mitochondrial suspensions to the cuvettes (succinate = 4 mM).

150  $\mu$ M ADP or ATP were added at the points indicated on the graph.





mitochondrial volume change. Figures 24 and 25 indicate that a change in osmotic concentration of the medium (from 0.1 M to 0.3 M sucrose) will result in a change in turbidity of the suspension of mitochondria ( 34  $\mu$ g mitochondrial protein per millilitre) of approximately 0.05 absorbance units. This corresponds to a change in volume of approximately 0.050 cubic microns (Figure 27.). From figure 37, we can determine that there is a change in absorbance of about 0.020 absorbance units after the second addition of ADP to the substrate containing mitochondrial suspension ( 140  $\mu$ g mitochondrial protein per millilitre). This turbidity change reflects a change in volume of approximately 0.005 cubic microns (0.020 absorbance units per 140  $\mu$ g mitochondrial protein, or 0.005 absorbance units per 34  $\mu$ g of mitochondrial protein). In the same figure, the second addition of ADP to a suspension containing the same amount of mitochondrial protein, but no substrate, caused a change in absorbance of about 0.036 units, corresponding to a change in volume of about 0.009 cubic microns. These calculations are based on the same assumptions stated on pages 164 to 169, namely that the procedure used for electron microscopic analysis of integrity results in fully shrunken mitochondria, and that these mitochondria are perfectly spherical. It



should be noted that at least 150  $\mu$ moles of ADP per millilitre of suspension was necessary to initiate the change in volume.

Age of tissue had no effect on the response to ethylene.

A series of inhibitors were tried. Azide, cyanide, and DNP blocked respiration linked mitochondrial volume changes, and induced a very slow swelling. Ouabain, a cardiac glycoside that affects adenosine triphosphatase activity in a number of tissues (Glynn 1964), was found to strongly inhibit the ethylene effect, at concentrations as low as 25  $\mu$ molar (Figure 38.). Considering that ouabain is not known to affect mitochondrial ATPase activity (Lehninger 1964), this was somewhat surprising.

Ethylene appears to affect the oxidative phosphorylation mechanism in an indirect manner. An enzyme involved in the mitochondrial volume changes regulated by ADP, and ATP would seem to be implicated. Such an enzyme, or enzyme system is the mitochondrial ATPase, which may be responsible for the transfer of energy to and from these molecules (Lehinger 1964, Cereijo-Santalo 1966a). The effect of ouabain would also







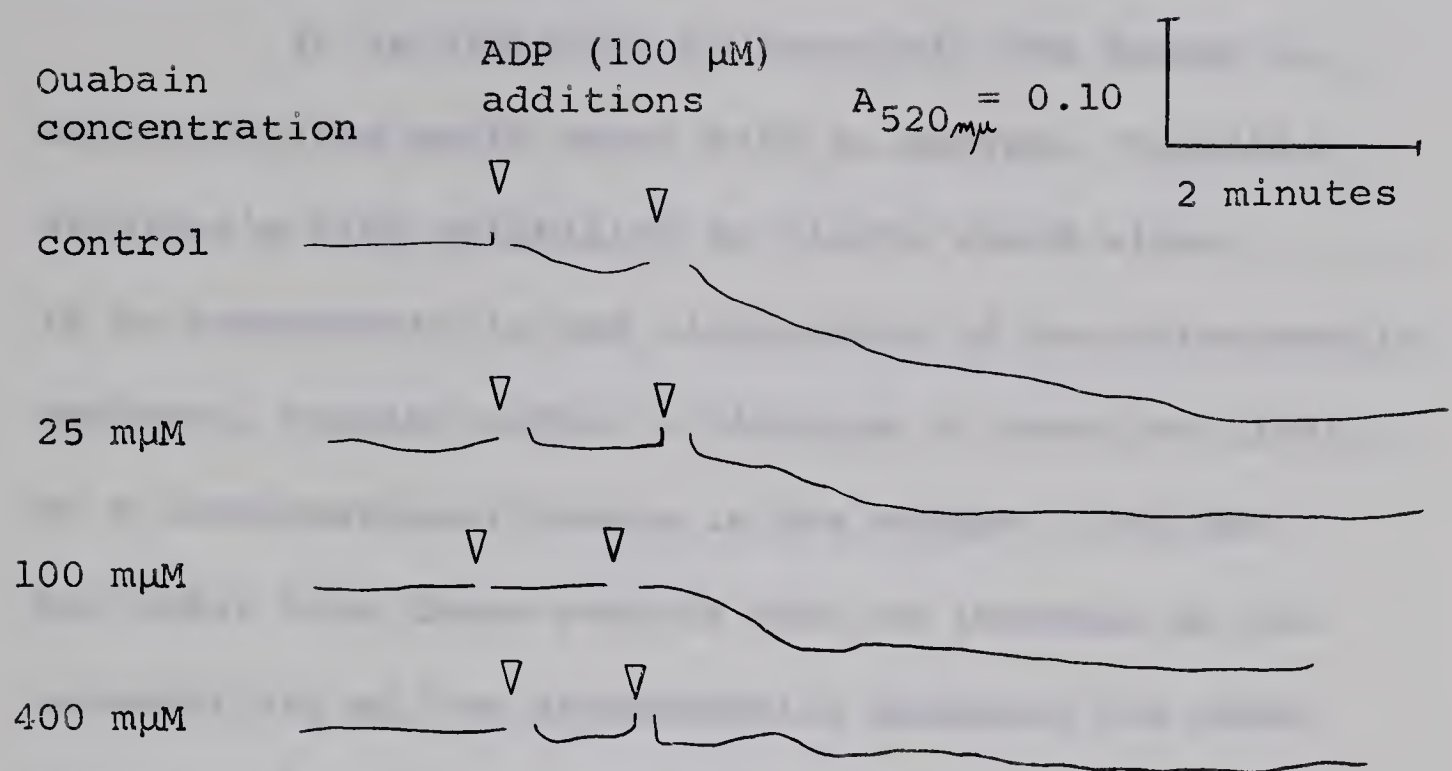


Figure 38. Differential spectrophotometry of bean cotyledon mitochondria (three day) treated with varying amounts of ouabain (as given), and 100 parts per million of ethylene. The assay medium contained 0.50 M mannitol, 0.01 M Tris-HCl, 0.001 M magnesium chloride, pH 7.4 at 25° C. The analysis was done at 25° C. Similar results with ATP addition were found.



implicate this enzyme system.

It is difficult to ascertain the manner in which ethylene would react with an enzyme. Possibly, ethylene's high solubility in lipids would allow it to concentrate in the lipoprotein of the mitochondrial membrane, causing either a blockage of reactive sites, or a conformational change in the enzyme. One can not infer from these results that an increase in the permeability of the mitochondrial membrane has taken place, as have Lyons, and Pratt (1964), but rather that an increase in the rate of transport, or uptake of ADP, and ATP has taken place under the influence of ethylene.





Rat liver mitochondria

A procedure closely related to that developed for the preparation of bean cotyledon mitochondria was devised after preliminary studies of the characteristics of rat liver mitochondria. The procedure used in these tests was based on that of Hogeboom, and Schneider (1952).

A Potter-Elvehjem homogenizer was used to fragment the rat liver in all of the preparations. This homogenizer was adequate, although quite slow to operate. Washing of the mitochondrial pellet was found to be essential because of contamination with blood and cellular debris.

Phase contrast, and electron microscopic analysis of the mitochondrial suspension prepared in the bean cotyledon media indicated that very little contamination was present.

Preparation in the standard bean cotyledon homogenization media was found to produce actively respiring mitochondria capable of oxidative phosphorylation. The bean cotyledon mitochondrial preparation, suspending, and assay media were used, for their use allowed easier comparison between the types of mitochondria studied, as well as simplifying solution



preparation.

Both Tris and Tricine were used in preliminary experiments. As no difference was found between the two buffers, Tris was used for all of the later studies. There was no problem in maintaining the pH of the suspension during homogenization, centrifugation, storage, and assay. Little change in respiratory control ratios were found over a period of three hours after the preparation of the mitochondrial fraction was completed. However, all analyses reported in this thesis were done within an hour after the completion of preparation. The quantity of mitochondria obtained was sufficient for extended experiments.

Respiratory control ratios ranging from five to greater than ten were found with the various substrates tested. In figures 39 to 41, which are summarized in table 5, the effects on mitochondrial respiration of additions of substrates are given. The amount of variation between preparations was far less than with the plant mitochondrial systems studied, indicating why most biochemists use rat liver as a source of mitochondria. Although the respiratory





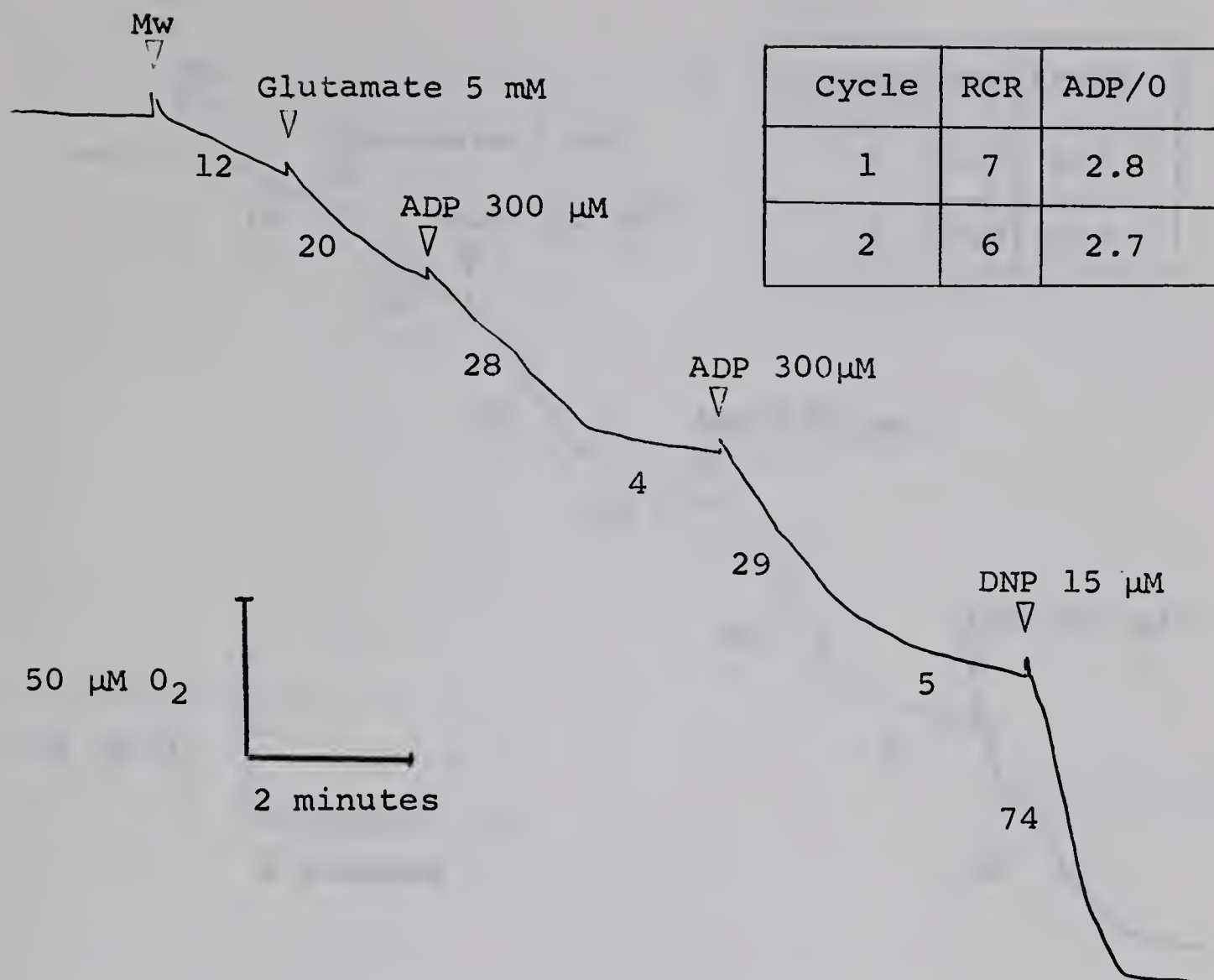


Figure 39. Polarographic trace of the oxygen uptake of mitochondria prepared from rat liver, and assayed in a medium containing 0.50 M mannitol, 0.01 M Tris-HCl, 0.001 M magnesium chloride, pH 7.4 at 25° C. 350 micrograms of mitochondrial protein were added per millilitre of assay medium, and the test was carried out at 25° C., using a Gilson Oxygraph. The numbers under the traces indicate the amount of oxygen uptake in  $\mu\text{moles}$  of oxygen per minute per millilitre of suspension.



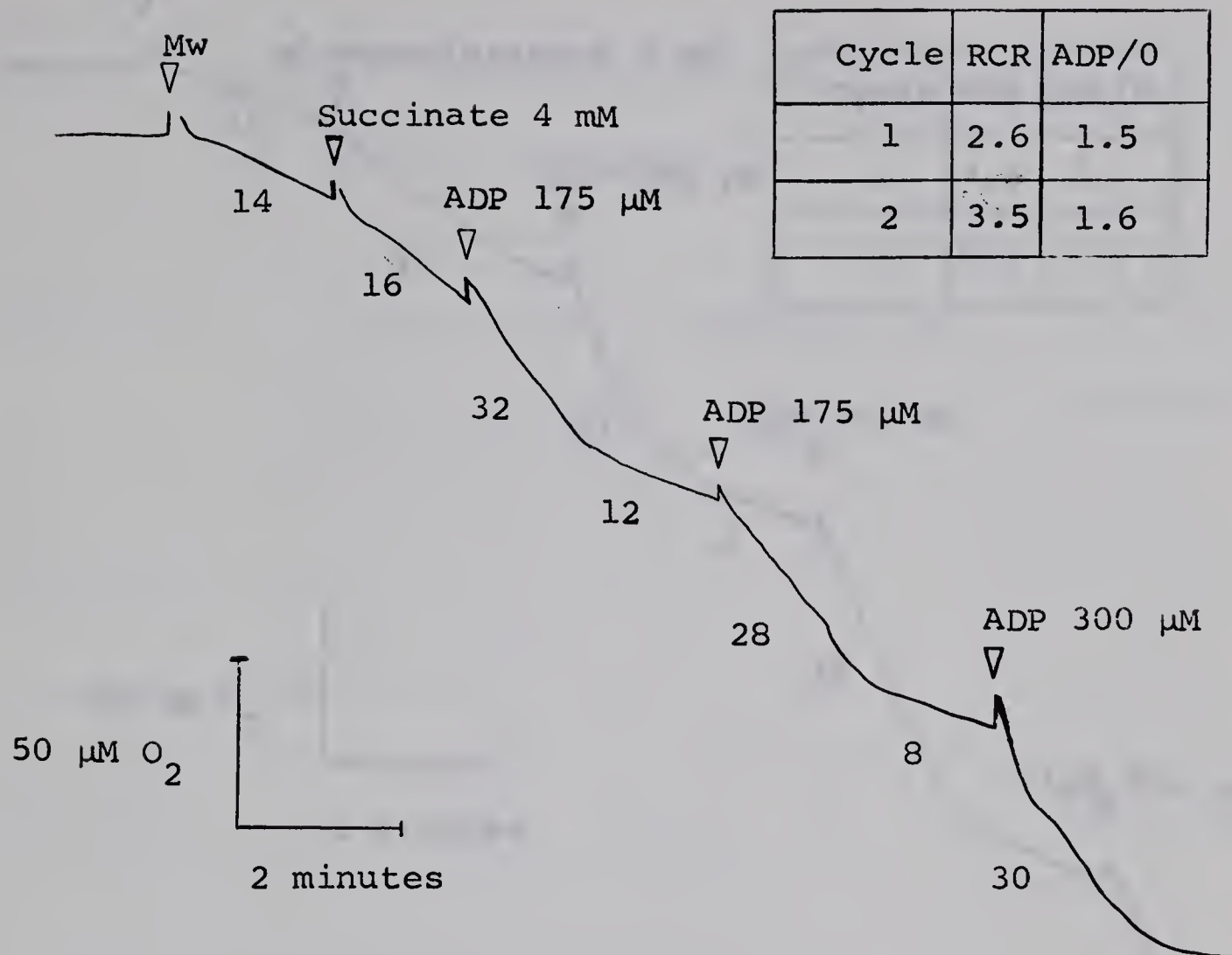


Figure 40. Polarographic trace of the oxygen uptake of mitochondria prepared from rat liver, and assayed in the standard assay medium, at 25° C. 350 micrograms of protein were added per millilitre of the assay medium, and the test was carried out with a Gilson Oxygraph. The numbers under the traces indicate the amount of oxygen uptake in  $\mu\text{moles}$  of oxygen per minute per ml. of suspension.





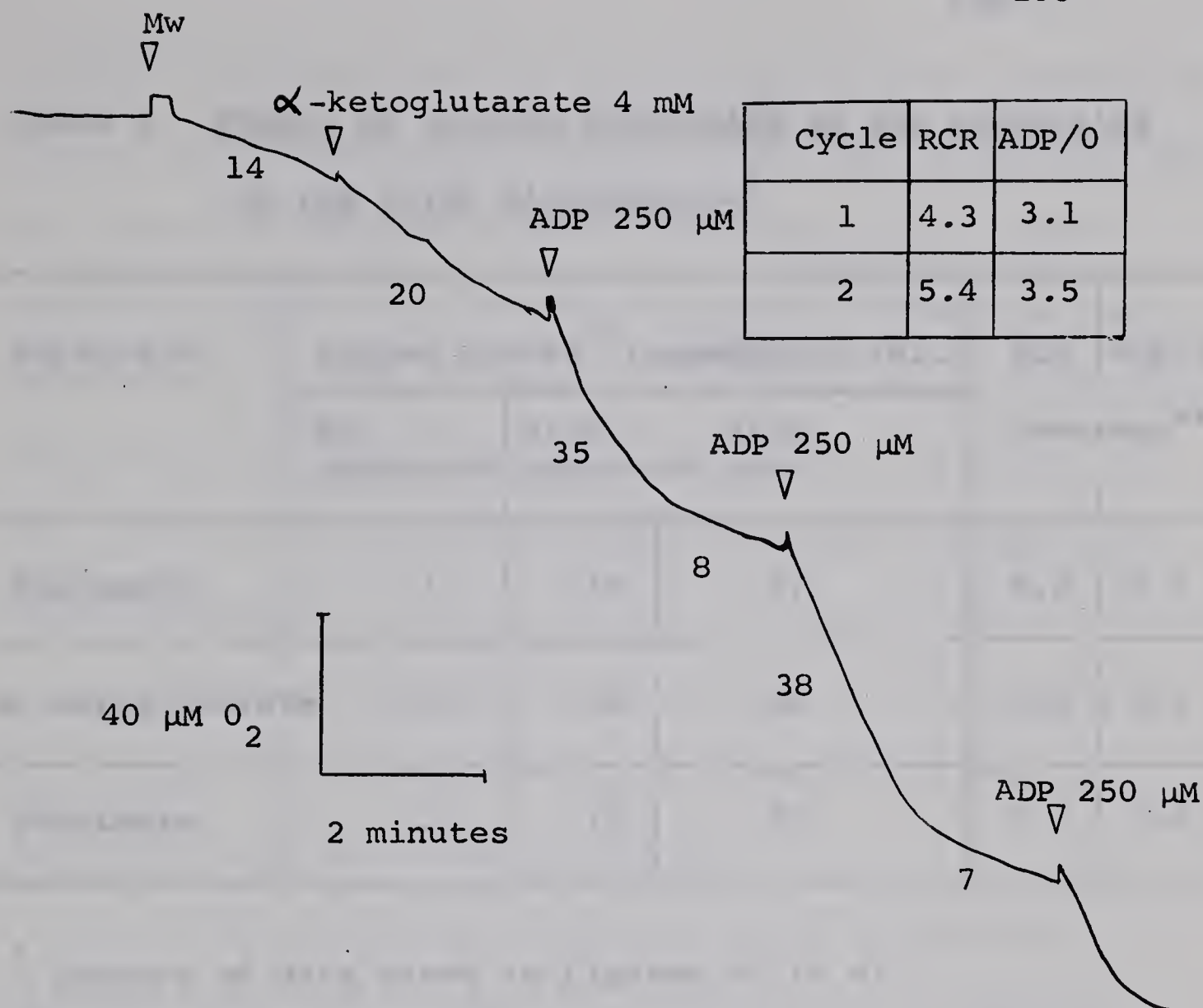


Figure 41. Polarographic trace of the oxygen uptake of a mitochondrial suspension prepared from rat liver, and assayed in the standard assay medium, at 25° C., using the Gilson Oxygraph. 400 micrograms of mitochondrial protein were added per millilitre of the assay medium. The numbers under the traces indicate the amount of oxygen uptake in  $\mu\text{moles O}_2 / \text{min.} / \text{ml.}$  of suspension.



Table 5. Effect of various substrates on the properties  
of rat liver mitochondria\*

Substrate	Oxygen uptake** (m $\mu$ moles/min./ml.)			RCR	ADP/O (average***)
	No substrate	With substrate	With ADP		
Glutamate	11	19	27	6.5	2.7
$\alpha$ -ketoglutarate	11	16	28	4.8	3.3
Succinate	13	15	37	3.0	1.6

\* Summary of data given in figures 39 to 41.

\*\* Oxygen uptakes have been corrected for the differences in the amount of protein added to each cuvette.

\*\*\* Average of respiratory control ratios, and ADP/O ratios for the cycles on each trace.





control ratios were not as high as reported for preparations done by more experienced workers, they were reasonably good. The extensive studies on the respiratory control characteristics of the rat liver mitochondrial suspensions were carried out, partly to determine the rat liver mitochondrial activity in the bean cotyledon media used, and partly to aid in the development of proper techniques for operation of the Oxygraph.

Passive changes in mitochondrial volume were not studied, as a large amount of research has been done on this topic (Tedeschi 1965).

Rat liver mitochondria in 0.15 M potassium chloride, and 0.50 M mannitol buffered media responded to swelling, and shrinking agents. Greater changes in volume were found in potassium chloride media, as expected, for mannitol is partially inhibitory towards large amplitude changes in volume in these mitochondria (Lehninger, Ray, and Schneider 1959). Calcium caused a gradual swelling, which could be reversed by the addition of ATP (Figure 42.). Phosphate had a similar effect, and its swelling was reversed by the addition of ADP, or ATP, or succinate (Figure 43.). The addition



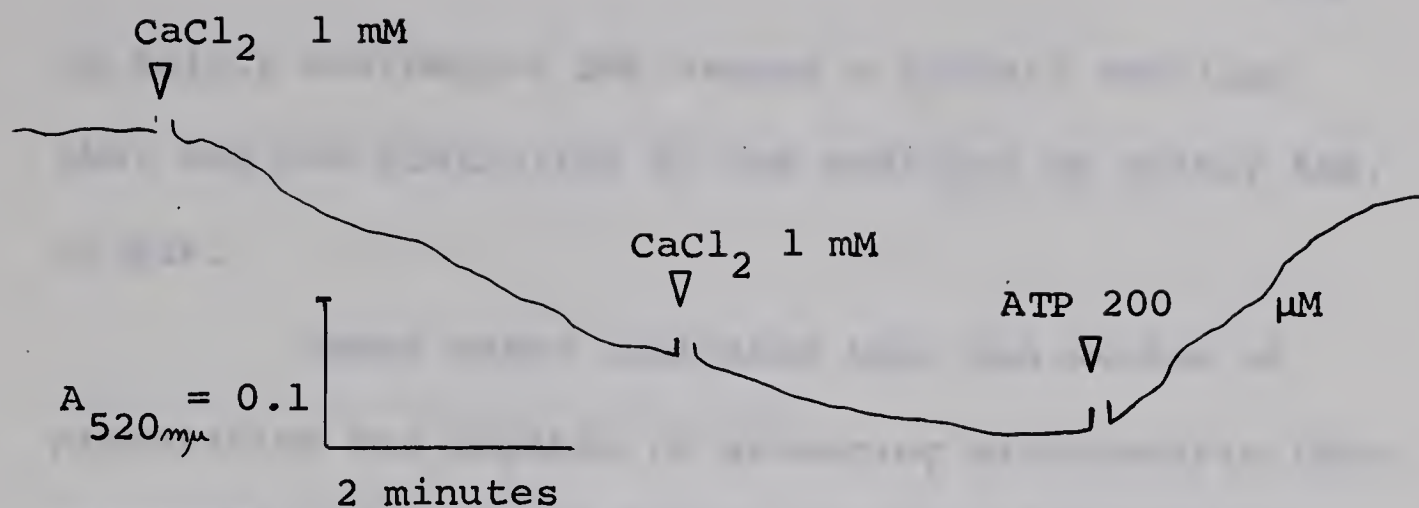


Figure 42. Changes in absorbance of a suspension of rat liver mitochondria (400 micrograms of mitochondrial protein per millilitre) on addition of calcium chloride and ATP. The standard assay medium plus 3 mM succinate was used, and the tests were done at 25° C.

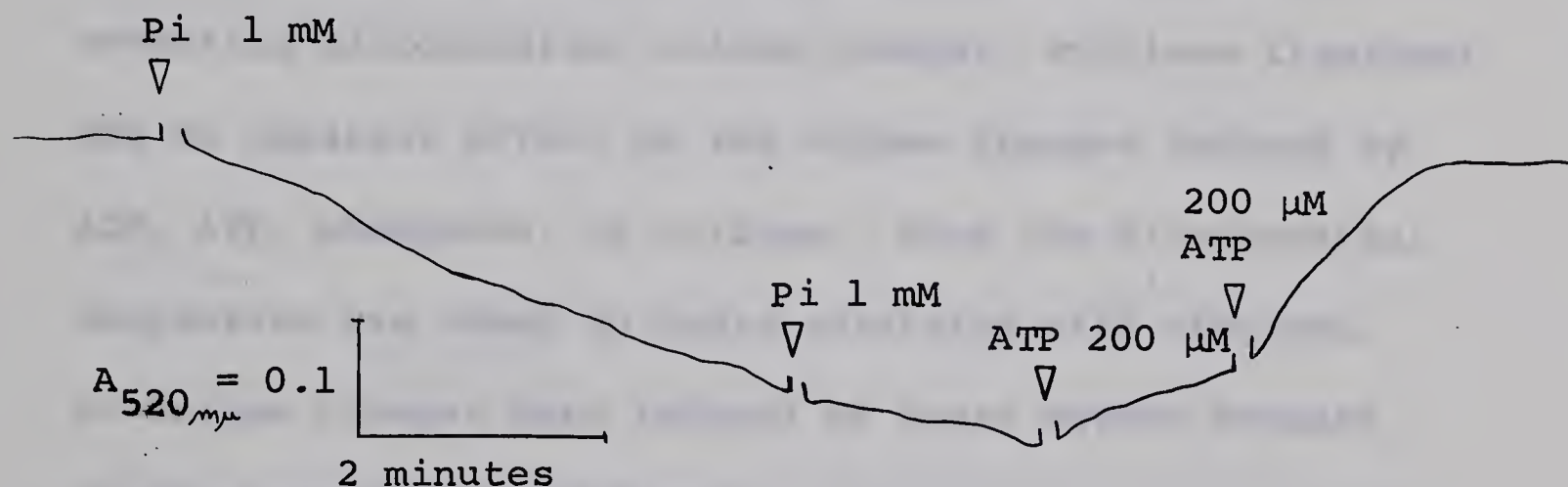


Figure 43. Changes in absorbance of a suspension of rat liver mitochondria (400 micrograms of mitochondrial protein per millilitre) on addition of phosphate (Pi), and ATP. The standard assay medium plus 3 mM succinate was used, and the tests were done at 25° C.





of thirty micromolar DNP caused a gradual swelling that was not controlled by the addition of either ADP, or ATP.

These tests indicated that the method of preparation was capable of producing mitochondria that responded in a similar manner to those discussed by other workers (Lehninger 1964).

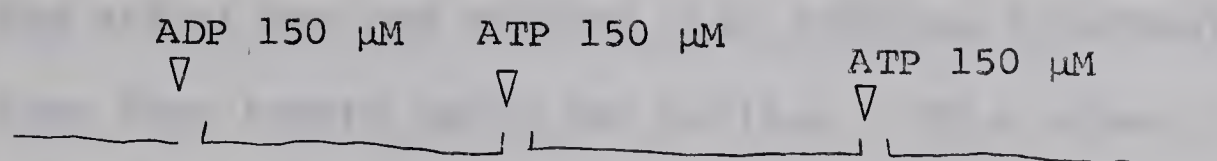
No effect of ethylene was found on oxygen uptake, respiratory control, or the phosphorylation of ADP, except at very high ethylene concentrations.

With the standard turbidimetric method of measuring mitochondrial volume changes, ethylene treatment had no apparent effect on the volume changes induced by ADP, ATP, phosphate, or calcium. When the mitochondrial suspension was added to media saturated with ethylene, no volume changes were induced by these agents because of the anaerobic conditions.

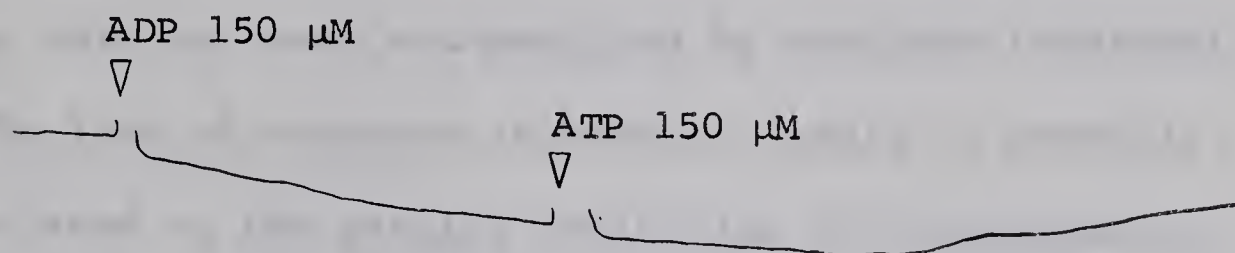
Initial tests with buffered 0.50 M mannitol media and with the spectrophotometer operated in a differential mode, indicated no difference between ethylene treated, and non-treated suspensions in terms of rates of mitochondrial volume changes. However, when 0.15 M potassium chloride was added to the media (Figure 44.), instead of mannitol, a definite increase in the rates



Control



100 parts per million ethylene



20 parts per million ethylene

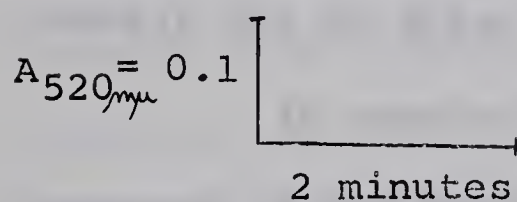
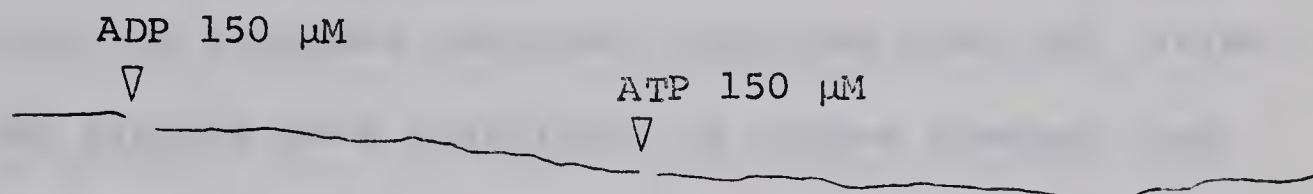


Figure 44. Differential spectrophotometry of rat liver mitochondrial suspensions. In each of the three experiments shown above, 300 micrograms of mitochondrial protein were added to each cuvette. The samples were made to a final volume of three millilitres with the standard assay medium. The suspension in one cuvette also contained the indicated amount of ethylene. The tests were done at 25° C.





of volume changes induced by ATP, and ADP, was noted. The effect was not evident with ethylene treatments of less than twenty parts per million. This effect is similar to that shown by Lyons, and Pratt (1964), although the rates of swelling induced by phosphate, or calcium, were not modified by ethylene treatment. The lack of response in mannitol media is probably related to the partial inhibition of mitochondrial volume changes by mannitol.

Study of the effects of various inhibitors upon the ethylene response indicated that DNP, azide, and cyanide were inhibitory to volume changes, and not specifically inhibitory to the ethylene effect. Ouabain had no effect.

In conclusion, rat liver mitochondria prepared in the bean cotyledon media system were fairly tightly coupled, and responded in a normal manner to various agents causing volume changes. Ethylene induced an increase in the rate of volume change caused by ADP, or ATP. The effect of ethylene upon animal mitochondria indicates a possible role for ethylene in the metabolic processes of animals. It is interesting to note that mitochondria prepared



from rat intestinal mucosa (Chandra 1963), and beef heart (Gibson 1963), have been shown to produce ethylene. It is thus possible that sufficient quantities of this gas may accumulate in animal tissues to affect mitochondria, and hence, cellular metabolism.

### Yeast mitochondria

Washing of the sample of yeast drawn from the culture unit was found to be essential, for dead cells in the suspension were evident as a gray layer on the surface of the pellet produced by the first centrifugation. The dead cells, when viewed under a phase contrast microscope were identifiable as such, because of a very dark outer membrane, and a homogeneous interior.

Employment of the Nossal cell disintegrator, or of long term sonication, to rupture the yeast cell walls, was discontinued because of the very low yields of mitochondria obtained. The cellulase procedure developed by Ohnishi, and Hagihara (1964) was found to be far easier to use, as well as giving a much better yield of mitochondria. Although the cellulase used would attack carboxymethyl cellulose





much more rapidly than yeast cellulose, it was sufficiently reactive to degrade portions of the yeast cell wall. (The yeast suspension was incubated for one and one-half to two hours at 30° C., with 500 milligrams of cellulase per 100 millilitres of suspension.) The catalytic action of the cellulase was aided by the thinner cell walls of yeast grown in continuous culture (Korn, and Northcote 1960).

The gradual formation of protoplasts during the incubation with cellulase was visible under a light microscope. A definite change in colour, and density of the incubation mixture was also evident.

The fluorometric procedure of Weber, and Laurence (1954) was tested with a graded series of dilutions of 0.2 % BSA, with each dilution containing 50 millimicromoles of ANS per millilitre. A linear relationship between the fluorescence of the dye, and the amount of protein in solution was shown. When aliquots of equal volumes of a yeast cell suspension were added to a similar set of solutions, no change in the relationship was found. This indicates that the fluorescent indicator (ANS) fluoresces only in the presence of protein.



The heat treatment destroyed the semi-permeable nature of the yeast cell, hence ANS was able to penetrate into the cell, and combine with cell protein. Since the cellulose cell wall appears to be the major barrier to the entry of the fluorescent indicator into the cell, the amount of cellulose degradation occurring in the yeast cell wall can thus be represented by the increase in fluorescence with time (Figure 45.).

Although this assay was originally developed for studies of bacterial permeability (Newton 1954), it could be used for other permeability assays, if used with care.

Yeast mitochondrial preparations appeared to contain a slight amount of fine debris. The mitochondria were spherical, with an average diameter of 0.50 microns. They seemed to be intact, although no internal structure could be seen in electron micrographs.

The homogenization, suspending, and assay media developed and used for bean cotyledon preparations, was used throughout the studies with yeast mitochondria, hence no conclusions as to their efficacy for yeast can be drawn. When one compares the results given here





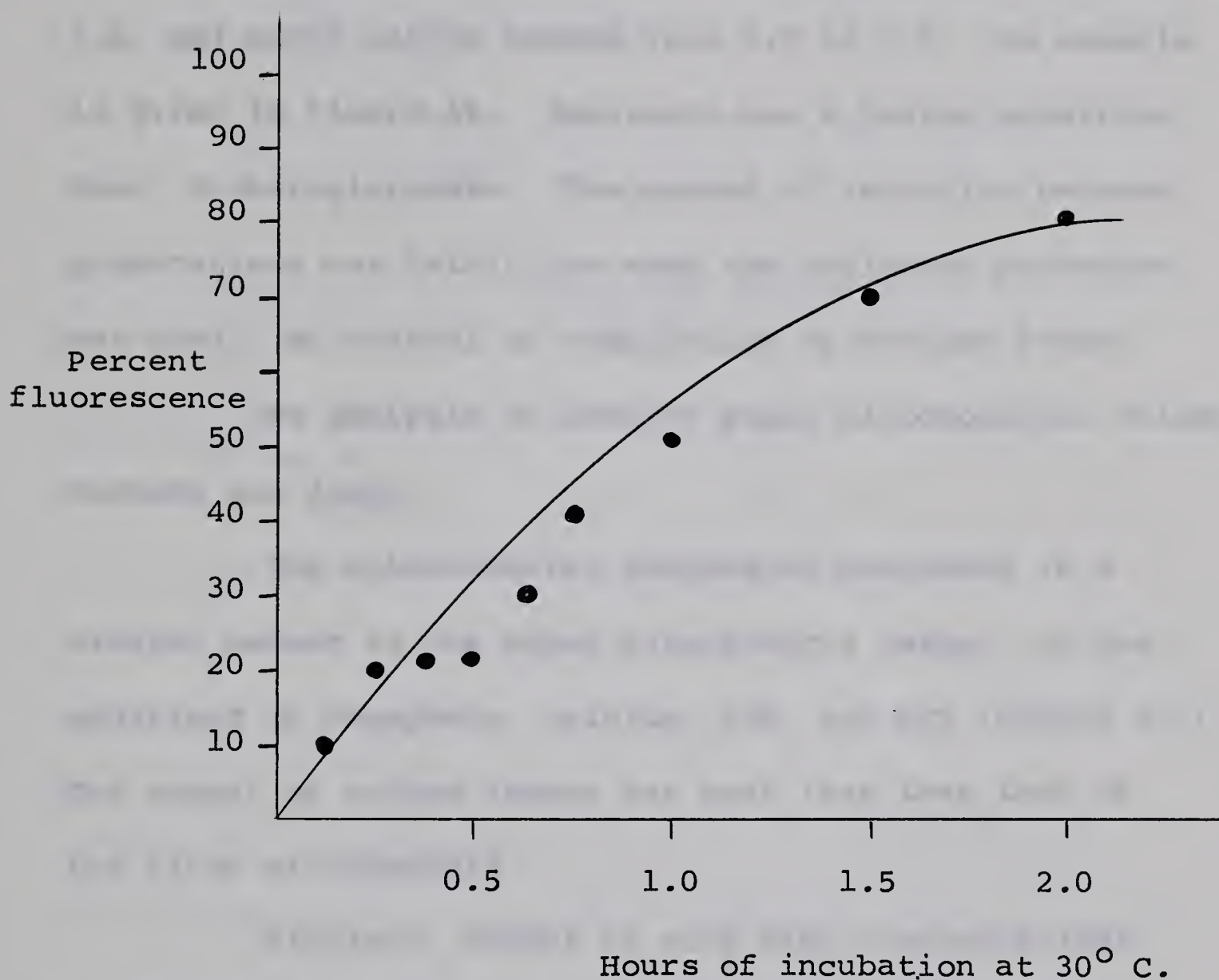
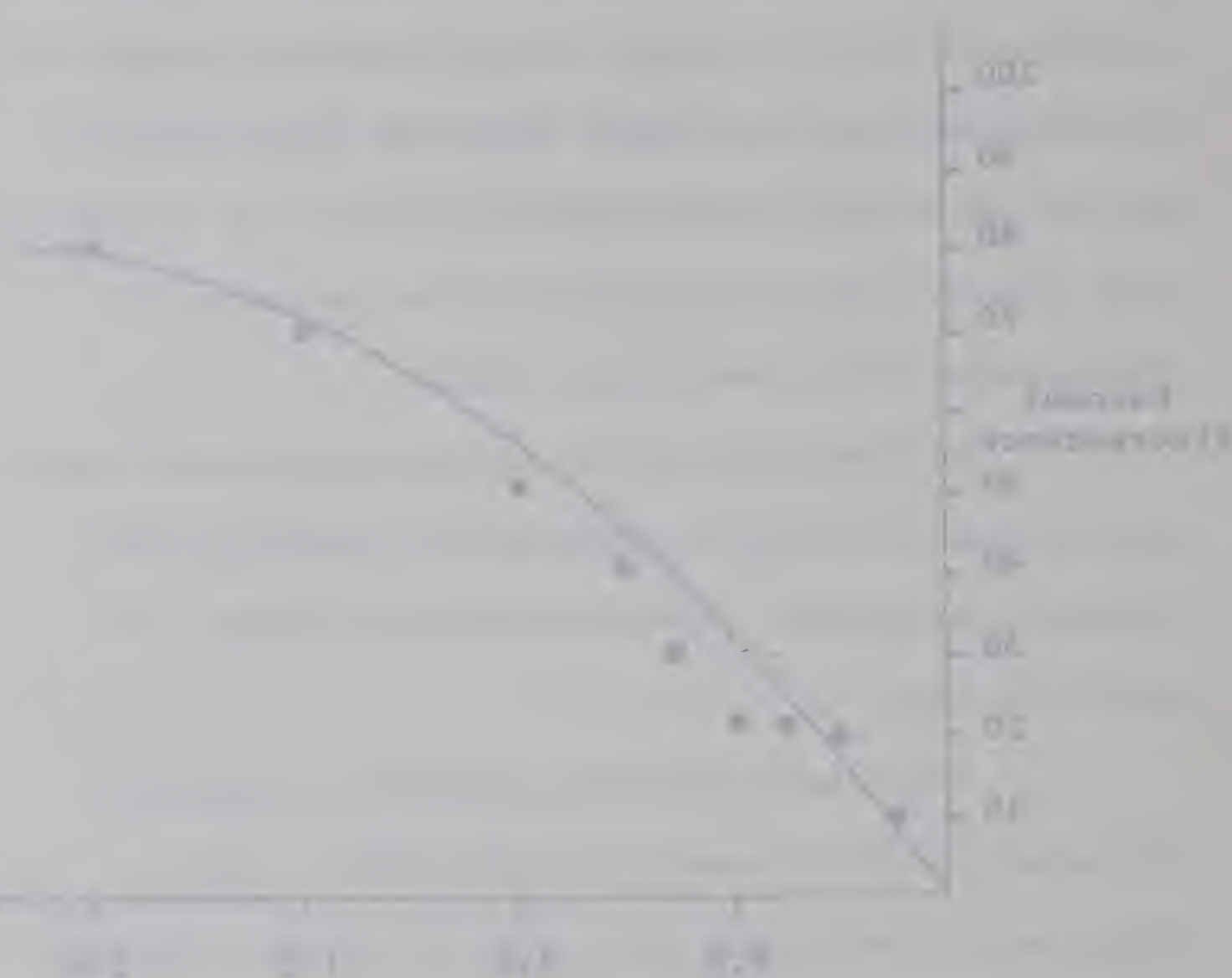


Figure 45. Measurement of the increase in fluorescence with time of a suspension of yeast treated with 0.5 % cellulase, and ANS. Zero fluorescence was set as the amount of fluorescence exhibited at zero time. 100 % fluorescence was set as that of the suspension after incubation at 95° C. for five minutes.



Amount of substance (g) is 0.9

Figure 1. Amount of substance (g) is 0.9

Amount of substance (g) is 0.9

Amount of substance (g) is 0.9

Amount of substance (g) is 0.9

Amount of substance (g) is 0.9

Amount of substance (g) is 0.9

with those of Ohnishi et al (1964), it is evident that not as active a preparation was developed.

Respiratory control ratios ranged from 1.1 to 1.8, and ADP/O ratios ranged from 0.5 to 0.9. An example is given in figure 46. Succinate was a better substrate than  $\alpha$ -ketoglutarate. The amount of variation between preparations was fairly low when the cellulase procedure was used. No control of respiration by ATP was found.

No analysis of passive yeast mitochondrial volume changes was done.

The mitochondrial suspension responded in a similar manner to the other mitochondria tested, to the additions of phosphate, calcium, ADP, and ATP (Figure 47.). The extent of volume change was much less than that of rat liver mitochondria.

Ethylene, except at very high concentrations such that anaerobic conditions were produced, had no apparent effect on mitochondrial respiration.

Differential spectrophotometry of yeast mitochondrial suspensions indicated that as little as twenty parts per million of ethylene would cause a





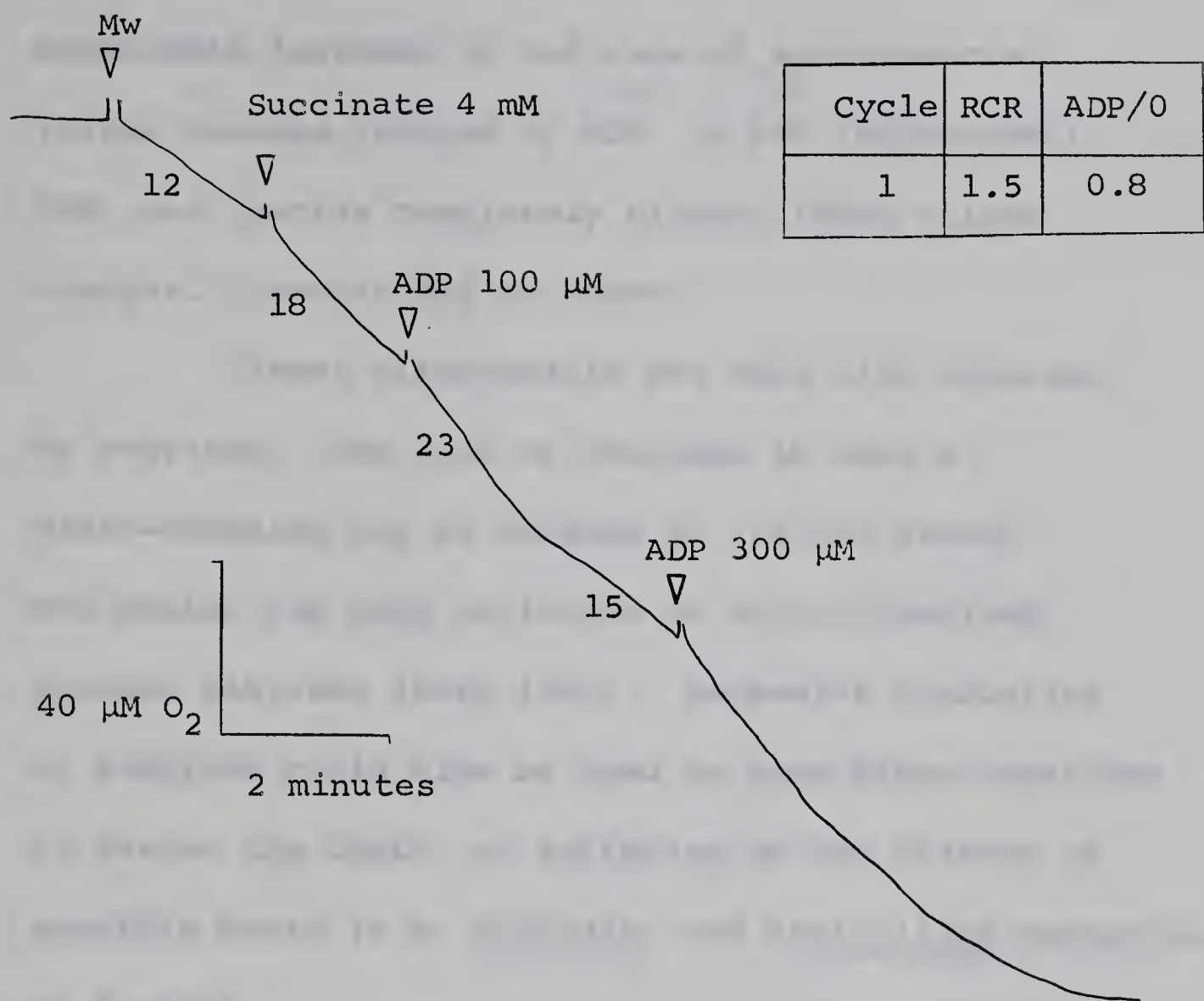


Figure 46. Polarographic trace of the oxygen uptake of a mitochondrial suspension prepared by the cellulase procedure from yeast. 400 micrograms of mitochondrial protein were added to a final volume of three millilitres of the standard assay medium, and the test was carried out with the Gilson Oxygraph, at 25° C.



measurable increase in the rate of mitochondrial volume changes induced by ADP, or ATP (Figure 48.). DNP, and cyanide completely blocked these volume changes. Ouabain had no effect.

Yeast mitochondria are thus also affected by ethylene. The role of ethylene in such a micro-organism may be related to its own growth, and death, for many varieties of micro-organisms produce ethylene (Burg 1962). Excessive production of ethylene could also be used by some micro-organisms to hasten the death, or softening of the tissues of possible hosts (e.g. Diplodia, and Penicillium infections of fruits).





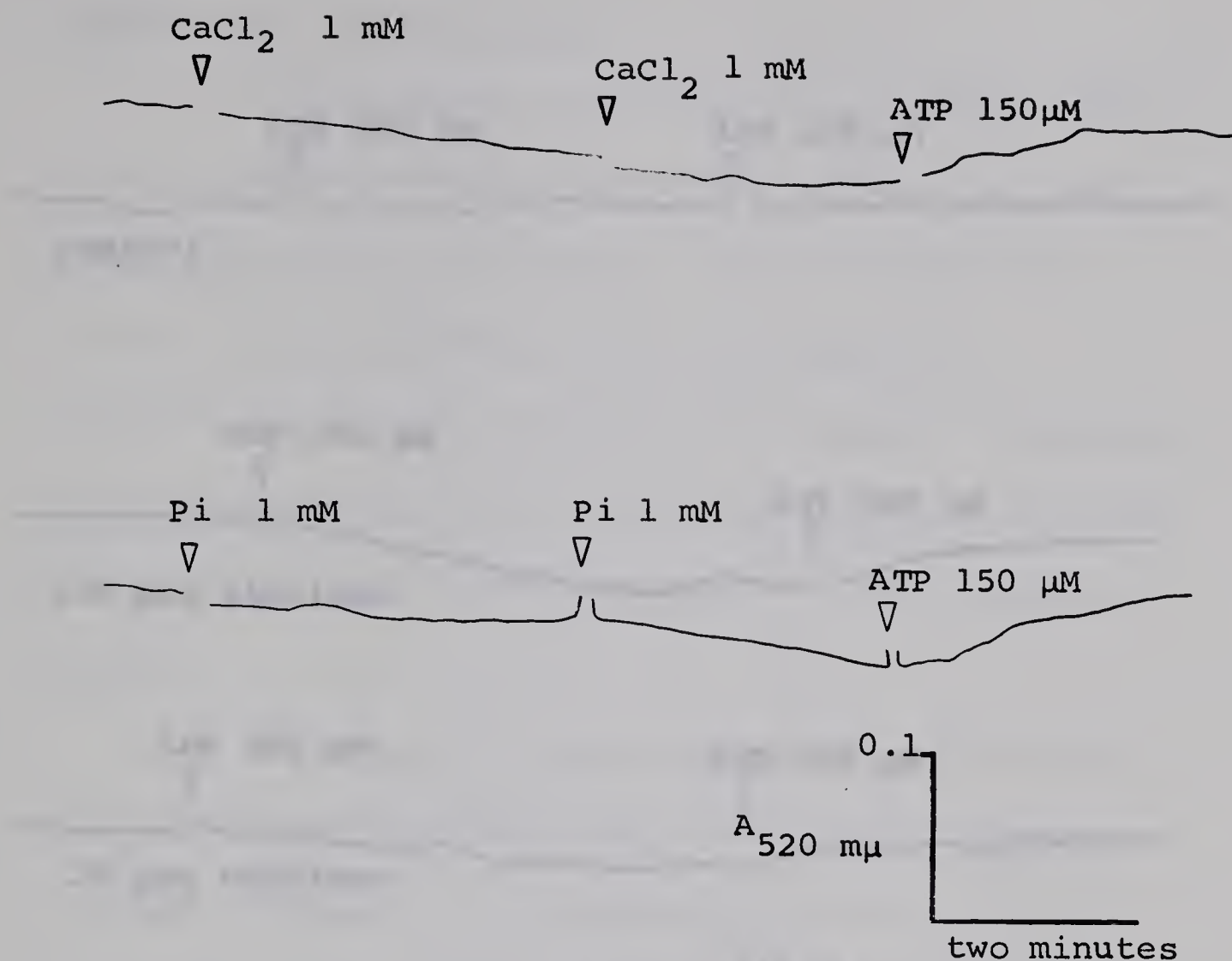


Figure 47. Change in absorbance of mitochondrial suspensions prepared by the cellulase procedure from yeast. The above experiments were done in the standard assay medium, at 25° C. 3 mM succinate was added prior to the beginning of the test. 350 micrograms of mitochondrial protein per millilitre was added to initiate the experiments.



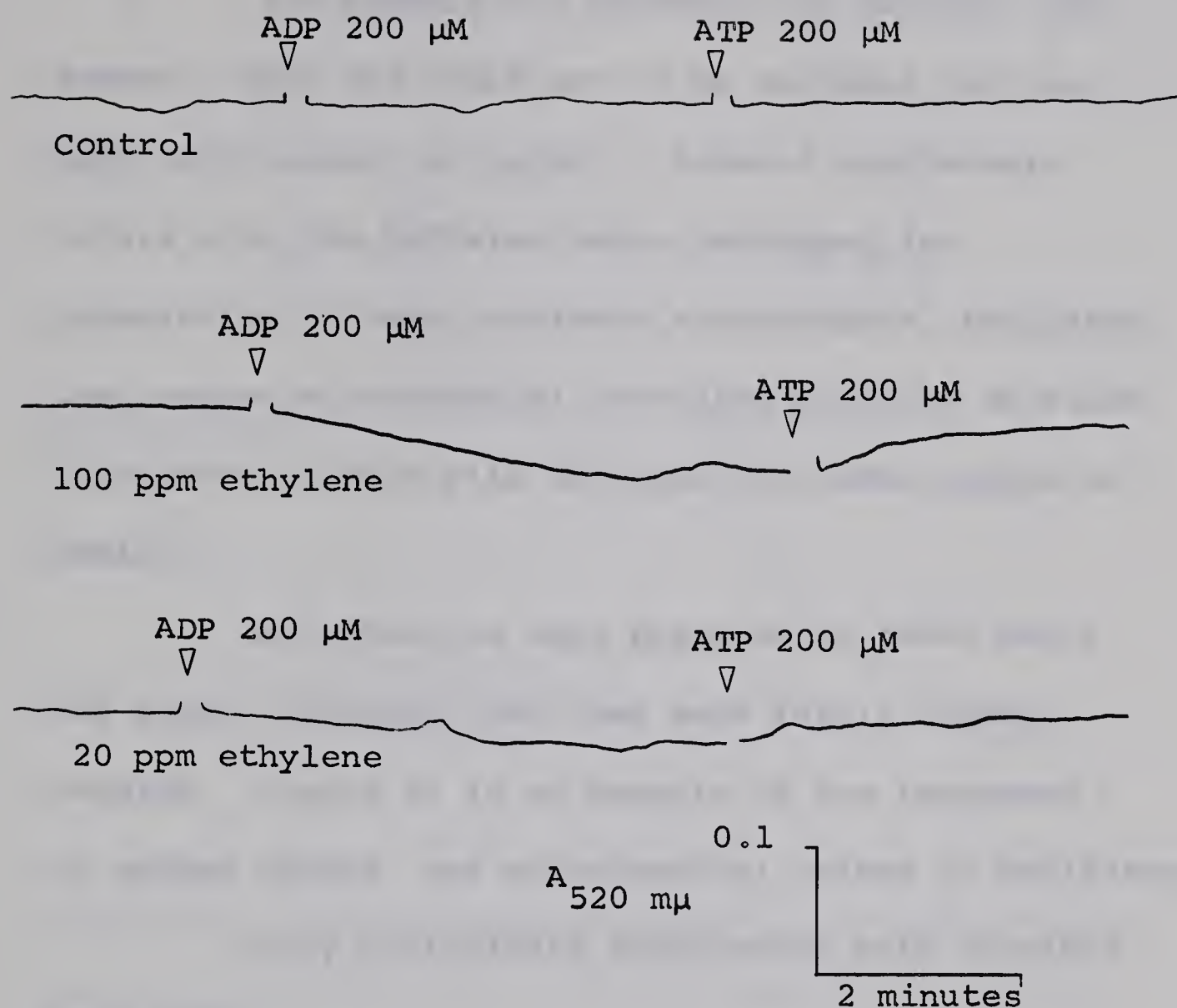


Figure 48. The effect of ethylene on the rate of ADP and ATP induced yeast mitochondrial volume changes. The standard assay medium with no added substrate was used for these experiments. All experiments were done at 25° C.





Potato tuber mitochondria

The preparation procedure of Wiskich, and Bonner (1963) was found not to be suitable for use with this variety of potato. Several preliminary trials with the buffered media developed for preparation of bean cotyledon mitochondria, indicated that active mitochondrial fractions could be obtained from potato tubers with the bean cotyledon system of media.

Mitochondria were prepared in these media, and assay indicated that they were fairly tightly coupled. Figure 49 is an example of the responses of oxygen uptake, and mitochondrial volume to additions.

Only preliminary experiments with ethylene were done.

The major problem with potato tuber tissue is the rapid darkening of homogenates. This apparent phenol oxidase activity may result in inhibition of mitochondrial respiration (Hulme, and Jones 1963). For this reason, the use of potato tubers was discontinued.



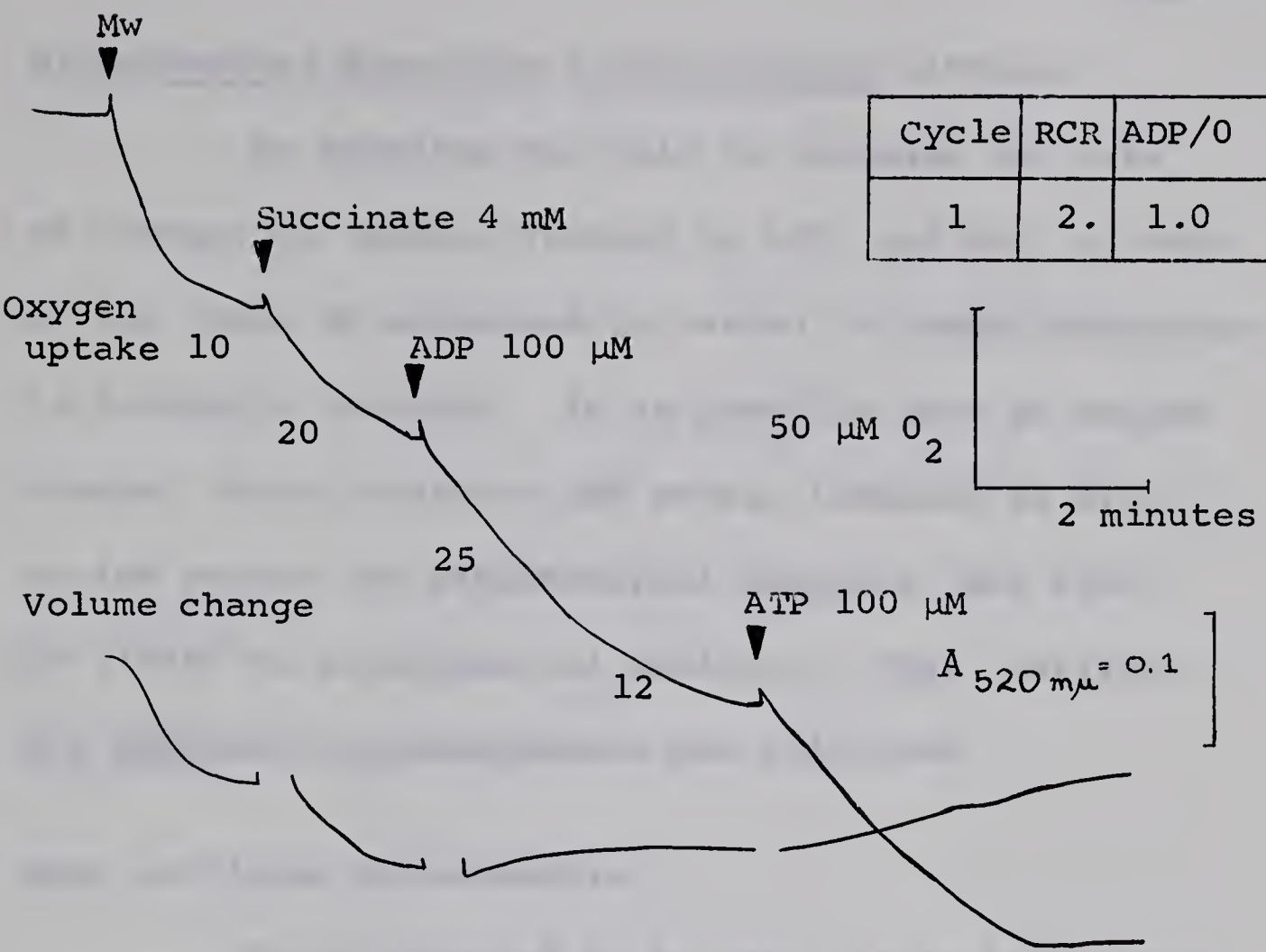


Figure 49. Simultaneous measurement of oxygen uptake, and absorbance changes of a mitochondrial suspension prepared from potato tubers. (300 micrograms of mitochondrial protein were added per millilitre of the assay medium.) The assay was conducted with the standard assay medium, in a final volume of three millilitres, using the polarographic system constructed for use in the spectrophotometer. The test was carried out at 25° c.





Mitochondrial adenosine triphosphatase (ATPase)

As ethylene was able to increase the rate of changes in volume, induced by ATP, and ADP, in each of the types of mitochondria tested, a common mechanism is evidently involved. It is possible that an enzyme system, which transfers the energy inherent in ATP, or ADP across the mitochondrial membrane, may also be linked to mitochondrial swelling. Thus, analysis for adenosine triphosphatase was initiated.

Bean cotyledon mitochondria

There appeared to be very little difference, in the ability to hydrolyze ATP, between mitochondrial preparations from different ages of bean cotyledons. Figure 50 indicates the effect of ethylene, and DNP treatment of three day bean cotyledon mitochondria, on the amount of phosphate produced by hydrolysis of ATP. No substrate was added, so as not to stimulate respiration, and oxidative phosphorylation.

Ethylene stimulated the hydrolysis of ATP. DNP had very little effect on the mitochondrial ATPase activity, although it caused a slight uncoupling when added to respiring mitochondria. In animal mitochondria, DNP causes a pronounced stimulation of



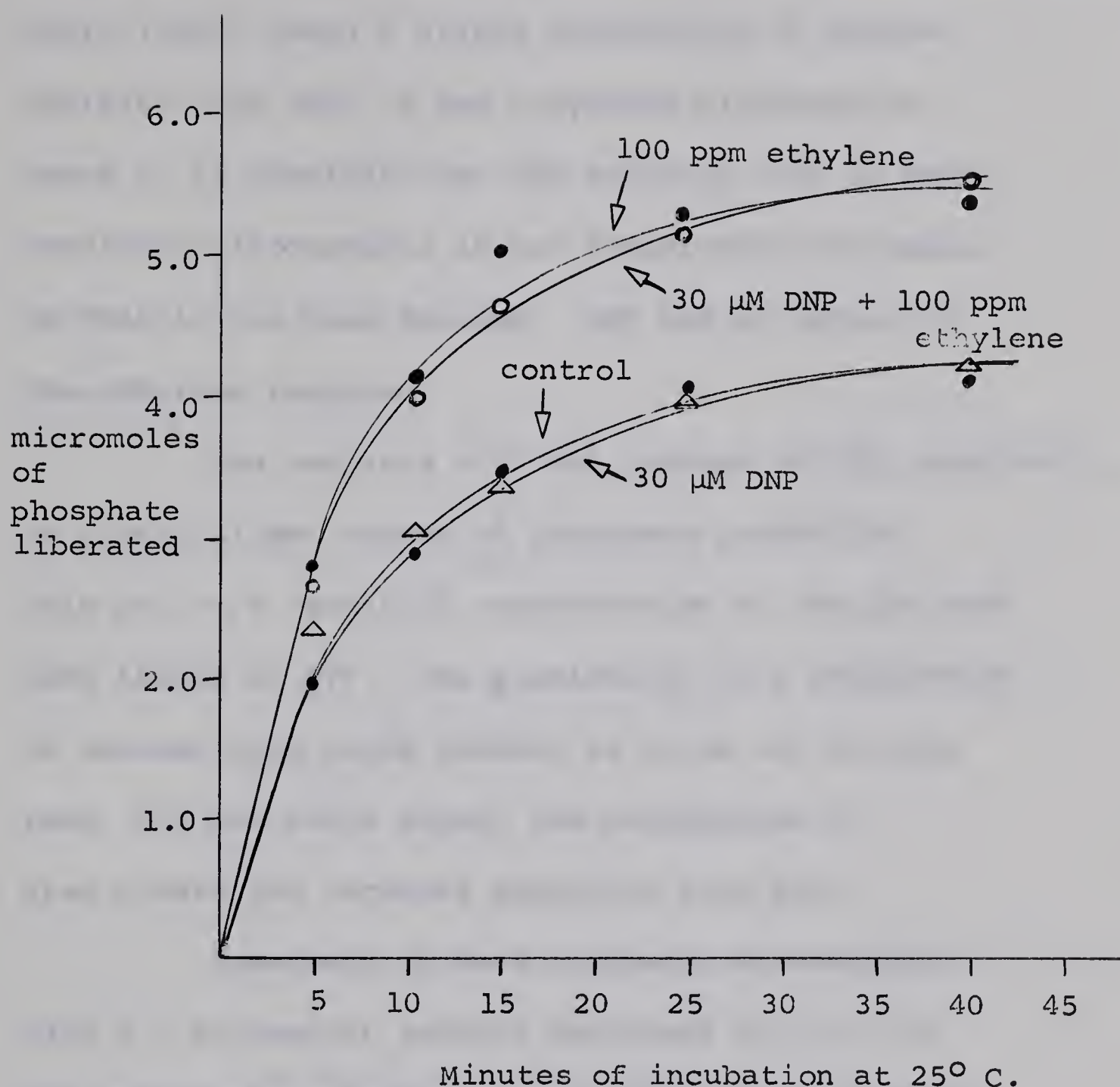


Figure 50. Measurement of the amount of inorganic phosphate liberated by bean mitochondrial adenosine triphosphatase treated with 100 parts per million of ethylene, and/or 30  $\mu$ M DNP. The medium consisted of 0.50 M mannitol, 0.01 M Tris-HCl, 0.001 M magnesium chloride, 100  $\mu$ M ATP, pH 7.4 at 25°C. 300 micrograms of mitochondrial protein were added per millilitre of medium.





ATPase activity, as well as of respiration (Lehninger 1964). Forti (1957) found a slight stimulation of ATPase activity with DNP, in pea cotyledon mitochondria, hence it is possible that DNP reactive site in bean cotyledon mitochondria is not immediately available, or that it has been damaged. DNP had no effect on the ethylene response.

One analysis with ADP instead of ATP, resulted in a very slight amount of phosphate production. This may be a result of contamination of the ADP used with traces of ATP. The possibility of a phosphatase of another type being present is ruled out by this test, for one would expect the phosphatase to also cleave the terminal phosphate from ADP.

Treatment of bean cotyledon mitochondria with 0.1 micromolar ouabain decreased the rate of ATP hydrolysis, whether or not ethylene was present. These results are shown in figure 51. This further corroborates the effect of ouabain on increases in the rate of volume change caused by ethylene. It is possible that this is a basic difference between plant and animal mitochondria, for Lehninger (1964) states, without qualification, that ouabain has not



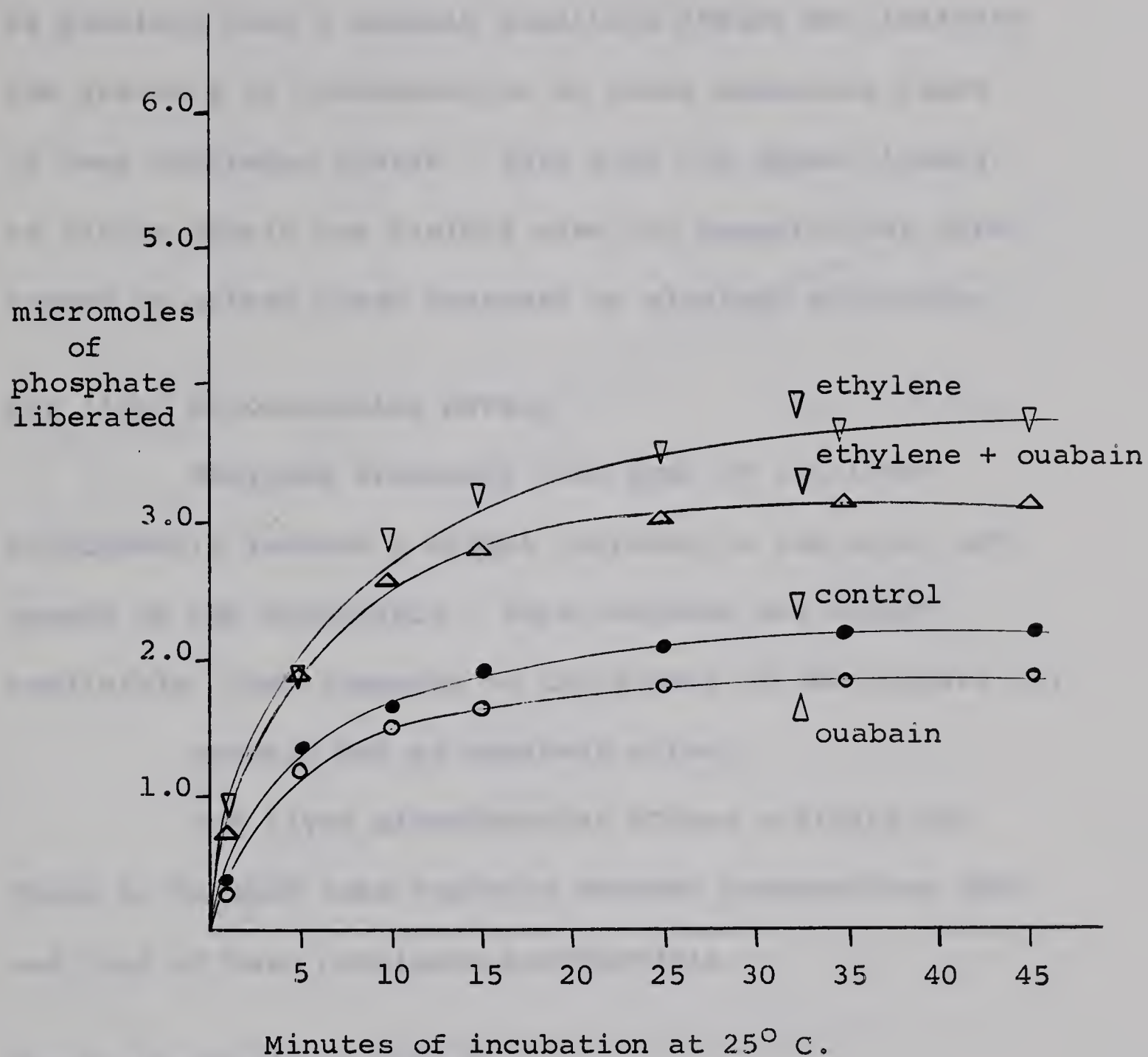


Figure 51. Measurement of the amount of inorganic phosphate liberated by bean cotyledon mitochondrial adenosine triphosphatase treated with 100 parts per million ethylene and/or 200  $\mu$ M ouabain. The medium consisted of 0.50 M mannitol, 0.01 M Tris-HCl, 0.001 magnesium chloride, and 100  $\mu$ M ATP, pH 7.4 at 25° C. 225 micrograms of mitochondrial protein were added per millilitre of medium.





have been found effective with animal mitochondria. It is possible that a ouabain sensitive ATPase may indicate the presence of contamination by other membranes found in bean cotyledon tissue. This does not appear likely, as little debris was visible when the preparations were viewed by either phase contrast or electron microscopy.

#### Rat liver mitochondrial ATPase

Ethylene treatment (100 ppm) of rat liver mitochondria induced a slight increase in the rate, and amount of ATP hydrolysis. This increase was almost negligible, when compared to the effect of DNP (Figure 52.).

Ouabain had no apparent effect.

Rat liver mitochondrial ATPase activity was found to be much less variable between preparations than was that of bean cotyledon mitochondria.

#### Yeast mitochondrial ATPase

DNP, and ethylene were only slightly stimulatory towards yeast mitochondrial ATPase (Figure 53.). This response corroborates the very slight effect of ethylene treatment on yeast mitochondrial volume changes (page 203).



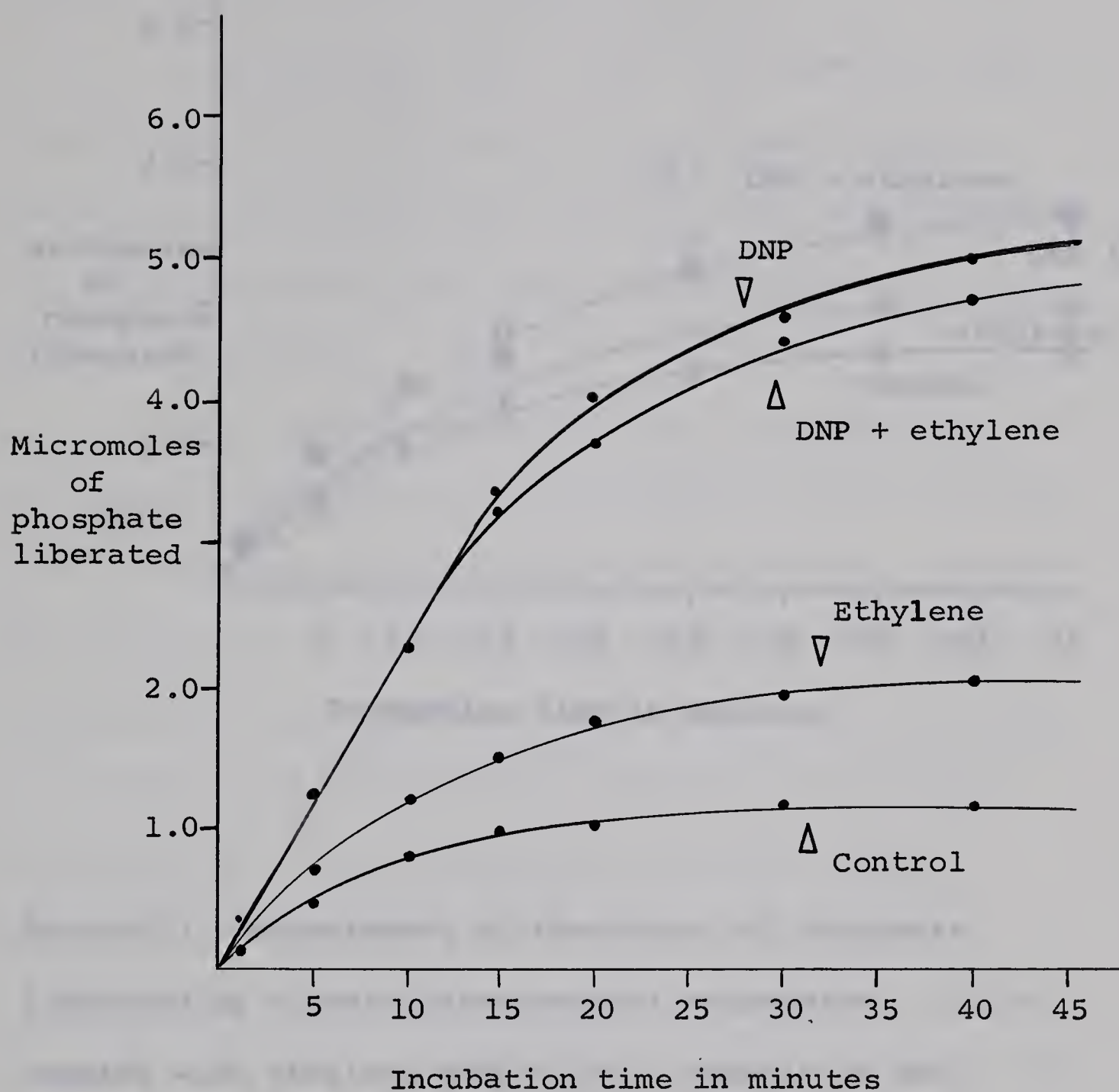


Figure 52. Measurement of the amount of phosphate liberated by rat liver mitochondrial suspensions treated with ethylene and/or DNP. Details of the method are given in figure 50. 300 micrograms of mitochondrial protein were added per millilitre of assay medium.





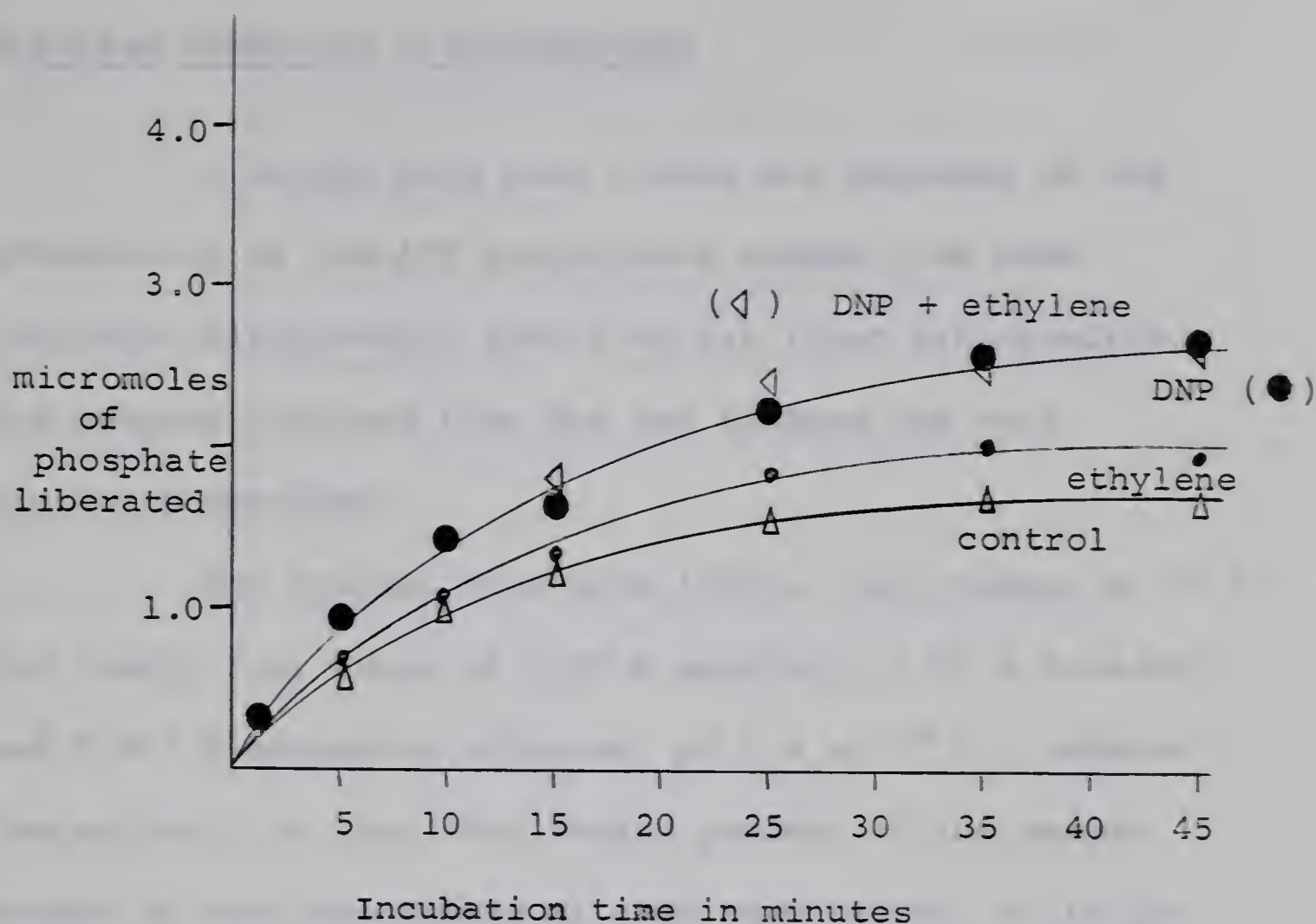


Figure 53. Measurement of the amount of phosphate liberated by a yeast mitochondrial suspension treated with ethylene and/or DNP. Details of the method are given in figure 50. 250 micrograms of mitochondrial protein were added per millilitre of the assay medium.

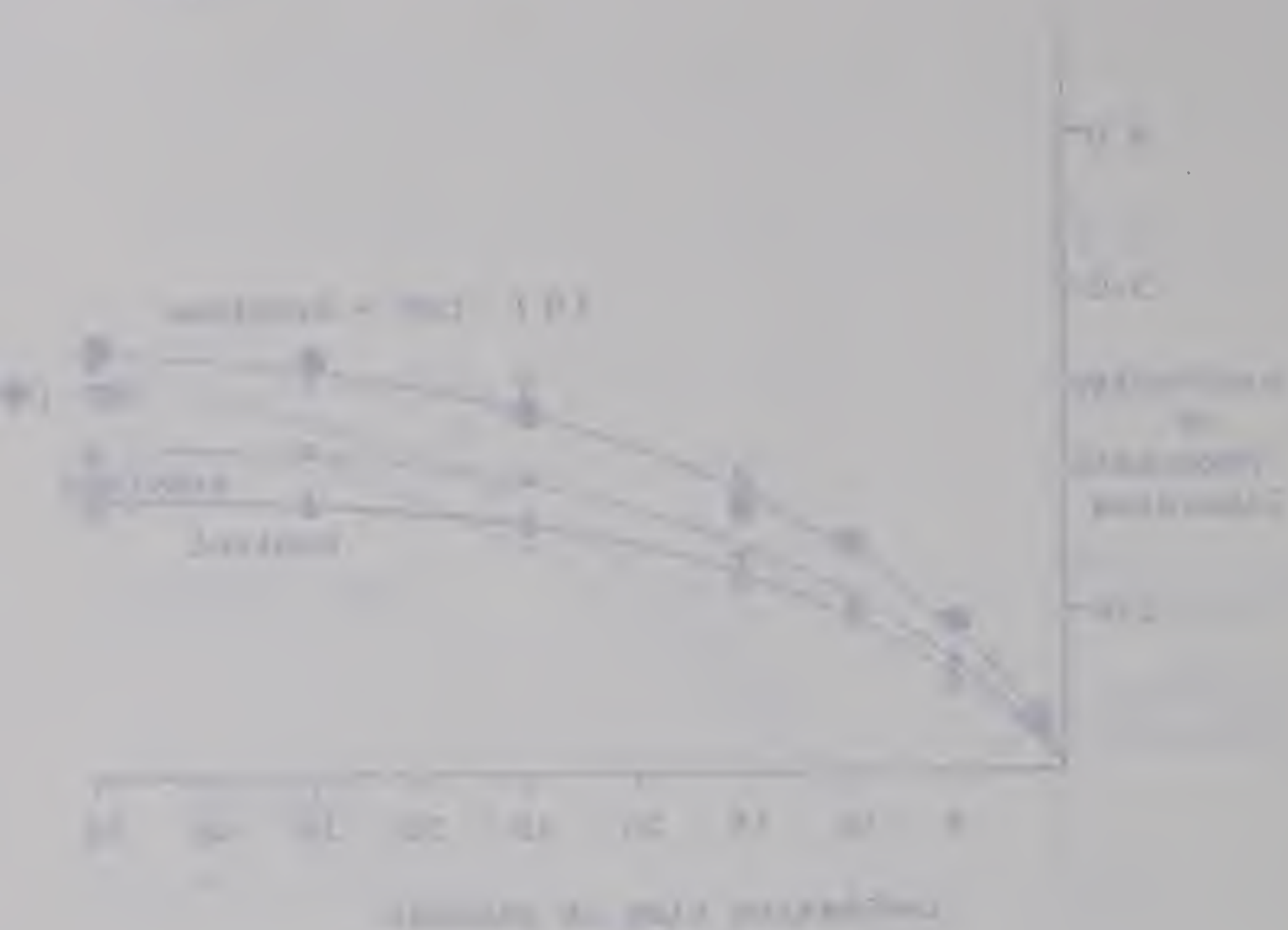


Figure 2.1 shows the effect of temperature on the rate of reaction. The graph shows that as the temperature increases, the rate of reaction also increases. This is because the particles have more energy and are moving faster, so they are more likely to collide and react. The graph also shows that the rate of reaction increases with the concentration of the reactants. This is because there are more particles in a given volume, so there are more collisions and reactions.

Purified adenosine triphosphatase

Although much more tissue was expended in the preparation of the ATP hydrolyzing enzyme from bean cotyledon mitochondria than from rat liver mitochondria, the enzymes produced from the two tissues had very similar properties.

The enzymes were cold labile, for storage at  $0^{\circ}\text{C}$ . for twenty four hours in 0.50 M mannitol, 0.01 M Tris-HCl, and 0.001 M magnesium chloride, pH 7.4 at  $0^{\circ}\text{C}$ ., reduced the activity to less than twenty percent of the enzyme stored in the same medium at room temperature, or in the same medium with fifty percent saturation of ammonium sulfate at  $0^{\circ}\text{C}$ . The particulate fraction did not appear to be affected by storage at  $-20^{\circ}\text{C}$ .

The major difference noted between the enzymes prepared from the two different sources was that the bean cotyledon mitochondrial enzyme preparation was not completely precipitated after two additions of protamine sulfate solution, whereas the rat liver mitochondria enzyme preparation appeared to come down primarily in the first precipitation.

Tables 6 and 7 give the yields and activities





of the preparation made from bean cotyledon mitochondria, and one of the preparations made from rat liver mitochondria, respectively.

The particulate system alone exhibited negligible ATPase activity. Lack of sufficient enzyme precluded study of the contention of Penefsky et al (1960) that addition of the enzyme to the particulate fraction produces a system capable of oxidative phosphorylation when substrates such as succinate are added.

With both types of preparations, no effect of ethylene was found. One hundred parts per million, or saturated solutions of ethylene, even after incubation for one-half hour at 25° C. had no effect on the rate of hydrolysis of ATP. Similarly, when the enzymes were added to their respective particulate fractions, no effect of ethylene was found.



Table 6. Bean cotyledon mitochondrial soluble ATPase

Step in procedure	Volume of material containing the ATPase	Protein (mg)	Total units*	Specific activity (units per mg protein)
Mitochondria	50	3420	-	-
Crude extract	60	420	450	1.07
Removal of isoelectric precipitate	55	240	350	1.49
Fractionation with ammonium sulfate	10	41	325	7.93
Treatment at 65° C.	4.5	18	1050	58.3

\* A unit of activity is defined as that amount of enzyme that catalyzes the turnover of 1.0  $\mu\text{M}$  of the substrate (NADH) per minute, under the conditions of the assay.





Table 7. Rat liver mitochondrial soluble ATPase

Step in procedure	Volume of material containing the ATPase	Protein (mg)	Total units*	Specific activity (units per mg protein)
Mitochondria	50	4100	-	-
Crude extract	80	490	440	0.89
Removal of isoelectric precipitate	75	165	310	1.88
Fractionation with ammonium sulfate	14	31	270	8.71
Treatment at 65° C.	3.5	14	930	66.4

\* A unit of activity is defined as that amount of enzyme that catalyzes the turnover of  $1.0 \mu\text{M}^{\text{ole}}$  of the substrate (NADH) per minute, under the conditions of the assay.



Alteration in the absorption spectra of the components  
of the mitochondrial media by ethylene

Ethylene may interact with the components of the mitochondrial media in such a way as to produce the effects discussed previously (effect of ethylene on mitochondrial volume changes, and ATPase).

Solutions containing components of the medium used in the preparation of bean cotyledon mitochondria were saturated with ethylene. Determination of the absorption spectra compared with a blank containing all of the components of the sample except for ethylene indicated a definite change in the absorption spectra in the presence of the gas.

Although ethylene itself absorbs in the far ultraviolet (166 to 190 mμ) as do a number of the components of the medium, a shift in the difference spectra was found towards longer wavelengths (195 to 220 mμ) when ethylene was present. The greatest shift occurred in the solutions of the more highly ionized compounds (potassium chloride, EDTA, Tris-HCl, and magnesium chloride). This suggests that there may be a charge-charge interaction between ethylene and the ions in solution. It is also possible that the presence of





high concentrations of ions in the solution may disturb the hydration lattice surrounding ethylene.

Infra-red difference spectrophotometry was attempted, but the use of water as a solvent made measurements very difficult to analyze. The characteristic spectrum of ethylene did not appear to be modified by the components of the medium.



Simulation of the effect of ethylene on mitochondria

Several of the programs developed for simulation of enzyme mechanisms are given in the appendix. The "Regular Simulator" program was designed for storage on tape, with a choice allowed for various subprograms. Some of the subroutines available in this program were used for direct output of all calculations, for normalizing physical parameters, and calculation of sum of concentrations of the free and bound form of each chemical. Other subprograms were used to simulate additions of chemicals when a supply became exhausted. This program was capable of handling up to eighty differential equations.

The "Simple Simulator" program was found to be suitable only for mechanisms involving less than eight differential equations.

Both programs can be used only on an IBM 7040, but can easily be modified for use with other computers.

Programs were checked for accuracy of calculation using the Michaelis-Menten equations as given on page 67. The results obtained (Figure 54.) were identical to those shown by Walter, and Morales (1964),





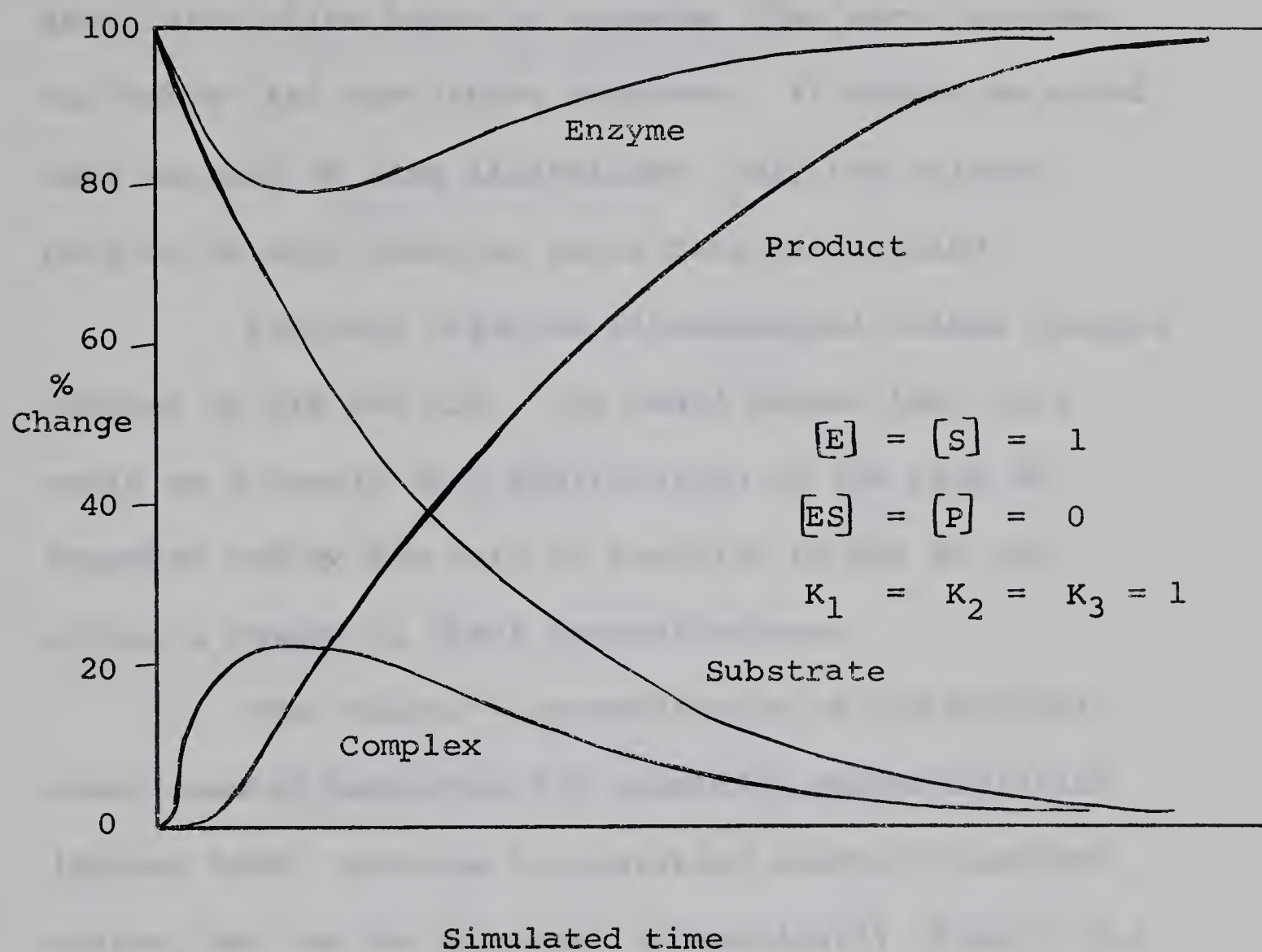


Figure 54. Simulation of the changes in concentration of the components of a single enzyme system. The mechanism, and the appropriate differential equations are given on page 67.



Hommes (1962), and Chance, Higgins, and Garfinkel (1963). About sixty-five hours of computer time were required to "debug" and test these programs. It should be noted that because of time limitations, only the initial portion of each reaction given here was studied.

Ethylene affected mitochondrial volume changes induced by ATP and ADP. One would expect that this would be a result of a modification of the rate of transfer and/or the rate of reaction of ADP or ATP, giving a change in their concentrations.

The "Slater" representation of the Mitchell chemi-osmotic mechanism for oxidative phosphorylation (Slater 1966) involves a simplified electron transfer system, and can be expressed schematically (Figure 55.) or as a set of equations (Figure 56). As with the other mechanisms studied, physical parameters were determined from the literature, and biochemical handbooks (Long, King, and Sperry 1961, Dawson, Elliot, Elliot, and Jones 1959). Typical parameters are given in tables 8 and 9. The differential equations corresponding to this mechanism are given in the appendix in computer format.

The effect of modifying the concentration within the normal concentration range (Chance 1964) of





Electron transfer

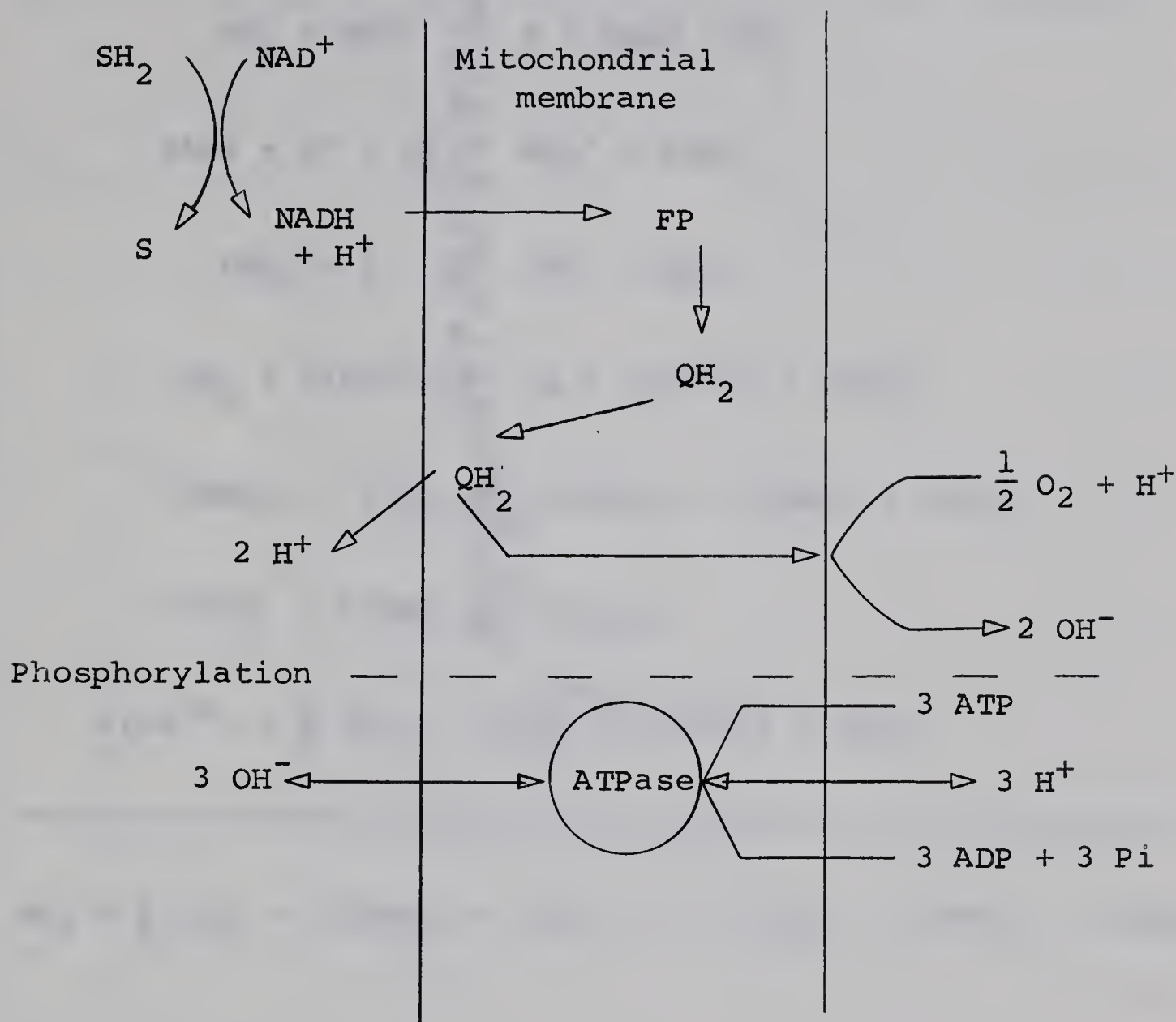


Figure 55. Schematic representation of the "Slater" mechanism. Details are given in figure 56, and in tables 8 and 9.



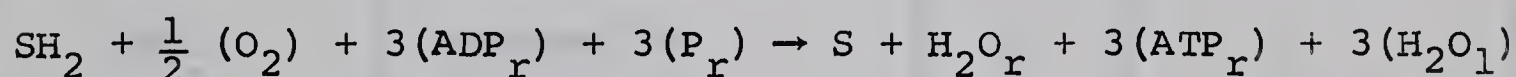
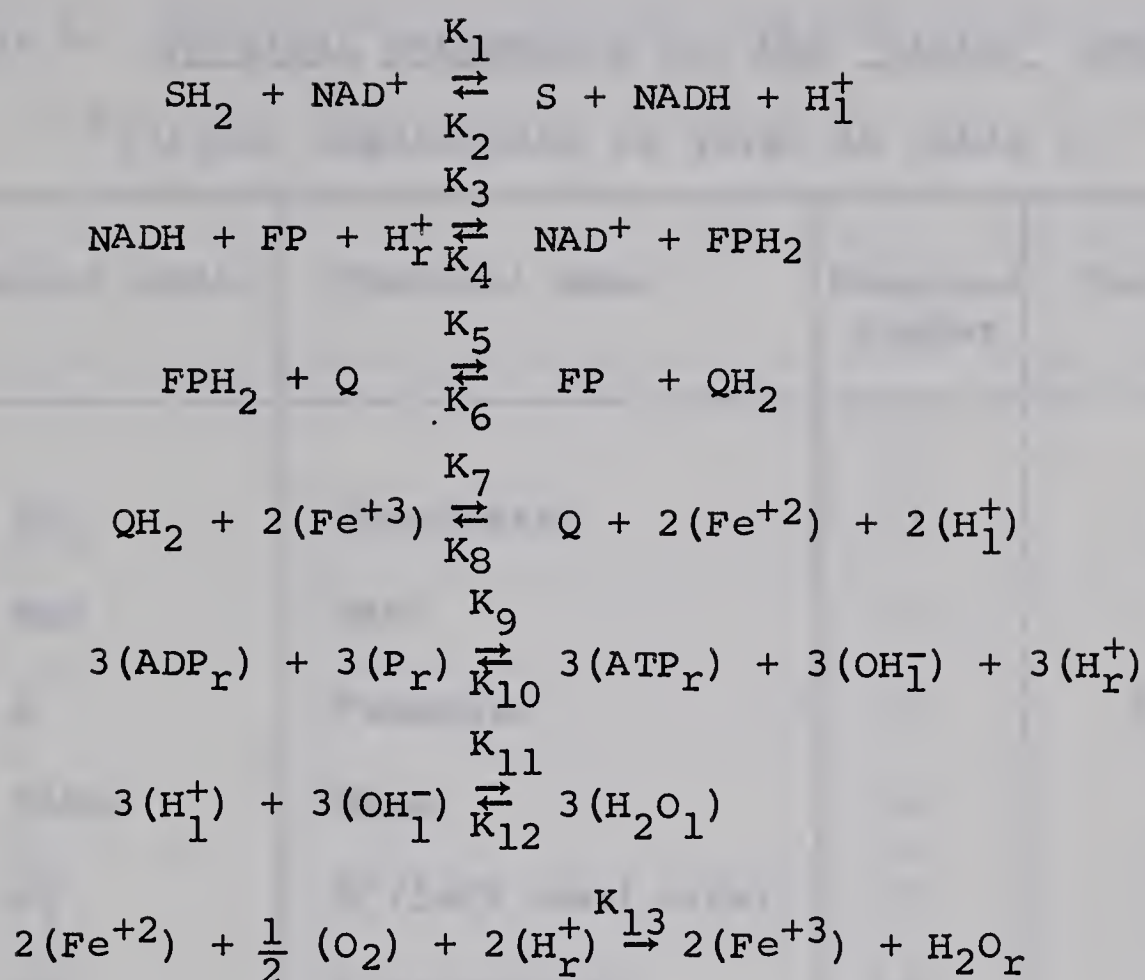


Figure 56. The "Slater" mechanism of oxidative phosphorylation. (Based on that given by Slater (1966))

The terms are defined in table 8.





Table 8. Physical parameters for the "Slater" mechanism\*

\*further explanation is given in table 9.

Chemical code	Chemical name	Chemical number	Concentration
SH <sub>2</sub>	Succinate	1	5.0 mμM
NAD	NAD <sup>+</sup>	2	2.0 mμM
S	Fumarate	3	20.0 mμM
NADH	NADH	4	1.0 mμM
H <sub>1</sub> <sup>+</sup>	H <sup>+</sup> (left hand side)	5	0.2 mM
FP	Flavoprotein	6	1.0 mμM
H <sub>r</sub> <sup>+</sup>	H <sup>+</sup> (right hand side)	7	0.2 mM
FPH <sub>2</sub>	Reduced FP	8	0
Q	Quinone	9	2.0 mμM
QH <sub>2</sub>	Reduced guinone	10	0.2 mμM
Fe <sup>+3</sup>	Cytochrome	11	2.0 mμM
Fe <sup>+2</sup>	Reduced Fe <sup>+3</sup>	12	0.2 mμM
ADP <sub>r</sub>	ADP	13	5.0 mμM
P <sub>r</sub>	Phosphate	14	234.0 mμM
ATP <sub>r</sub>	ATP	15	1.0 mμM
OH <sub>1</sub> <sup>-</sup>	OH <sup>-</sup> (left hand side)	16	0.2 mM
H <sub>2</sub> O <sub>1</sub>	H <sub>2</sub> O(left hand side)	17	200.0 mM
O <sub>2</sub>	Oxygen	18	500.0 mμM
H <sub>2</sub> O <sub>r</sub>	H <sub>2</sub> O(right hand side)	19	200.0 mM



Table 9. Typical rate constants for the "Slater" mechanism

Rate constant K ( )	Value of rate constant (mmoles per second)
1	0.150
2	0.010
3	70.0
4	1.0
5	4.0
6	1.0
7	160.0
8	5.0
9	0.05
10	0.25
11	200.0
12	0.01
13	1.0

The "Chemical number" given in figure 8, and the number of the rate constants given above refer to the equations in figure 56, and are used to identify the particular parameter used in the differential equations (see appendix).





ADP is shown in figures 57 to 59. Although electron transfer was alone sufficient to produce a charge separation between the "inner" and "outer" sides of the membrane to drive phosphorylation, a difference of about two pH units appeared to be essential if phosphorylation were to occur at the rate found in tightly coupled mitochondria (In figures 57 to 59, it can be seen that the hydrogen ion concentration "inside" the mitochondrion dropped rapidly about two pH units.) Present evidence indicates (Chance, and Mela 1966b) that such a gradient may be generated, although the largest pH difference found this far has only been of the order of one pH unit. As well, this gradient could only be generated by the uptake of calcium ions, for phosphate was found to rapidly collapse the gradient (Chance, and Mela 1966b).

Other workers (Mitchell 1966b) have shown that a gradient of 0.1 to 0.2 pH units can be generated during electron transfer. It is possible that such a charge is sufficient, with phosphorylation occurring in a rapid and cyclical manner. Chance, and Mela (1966a) have shown by experiment that the pH gradient across the mitochondrial membrane collapses very rapidly. This suggests that electron transfer may generate such



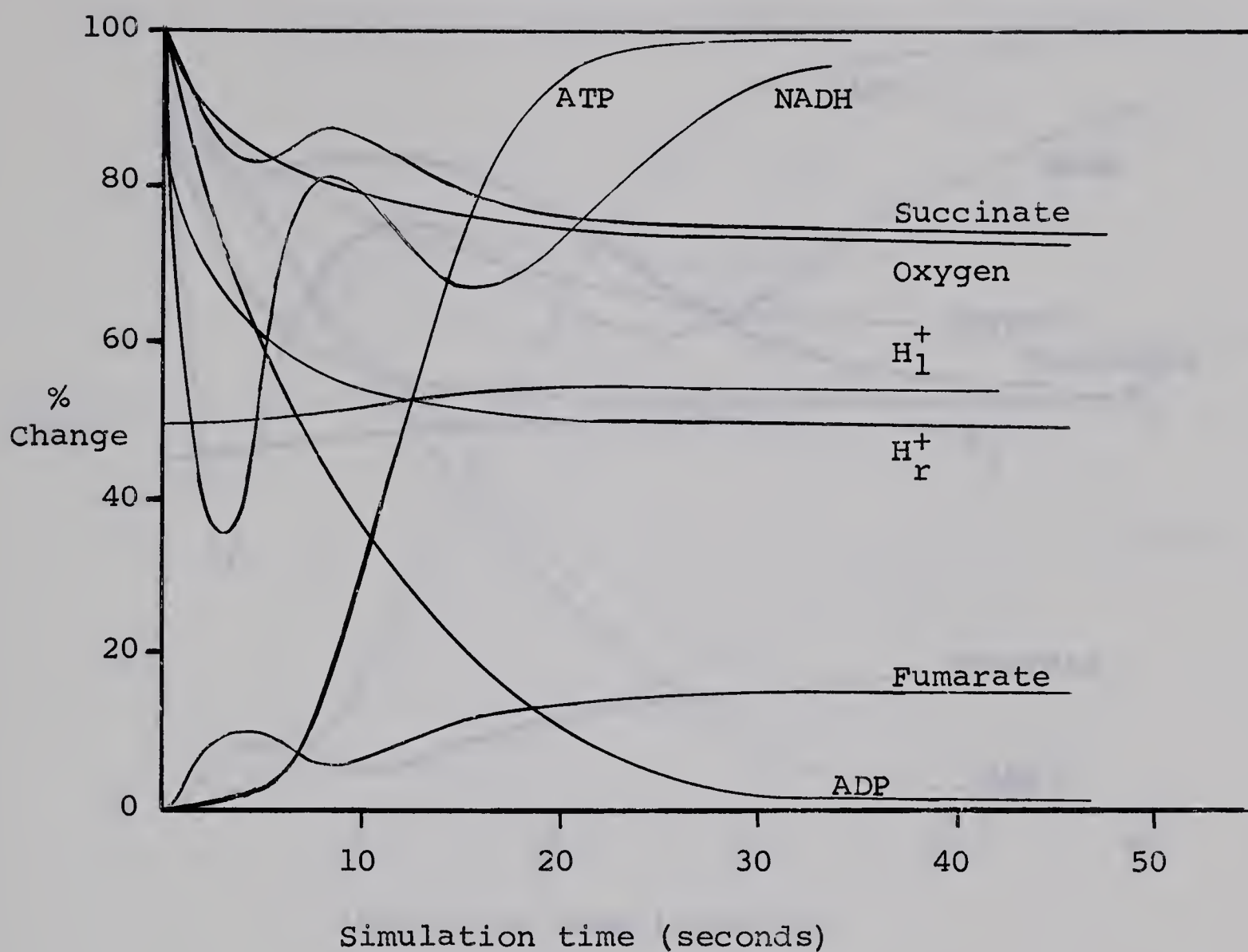


Figure 57. Changes in concentration of the components of the "Slater" mechanism of oxidative phosphorylation. The initial concentration of ADP in this test was .05  $\mu$ molar.





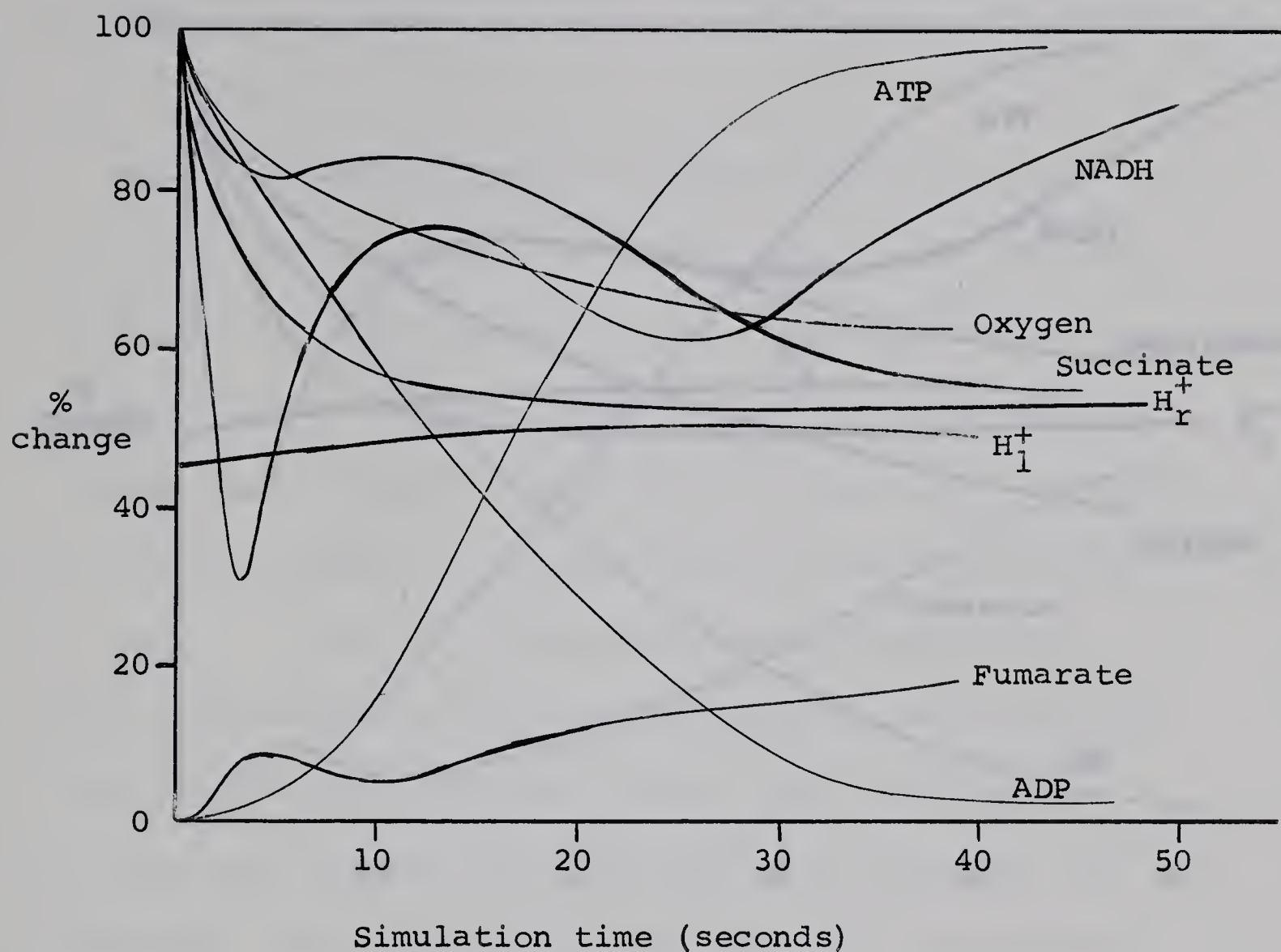


Figure 58. Changes in concentration of components of the "Slater" mechanism of oxidative phosphorylation. The initial concentration of ADP used in this test was 0.5  $\mu$ molar.



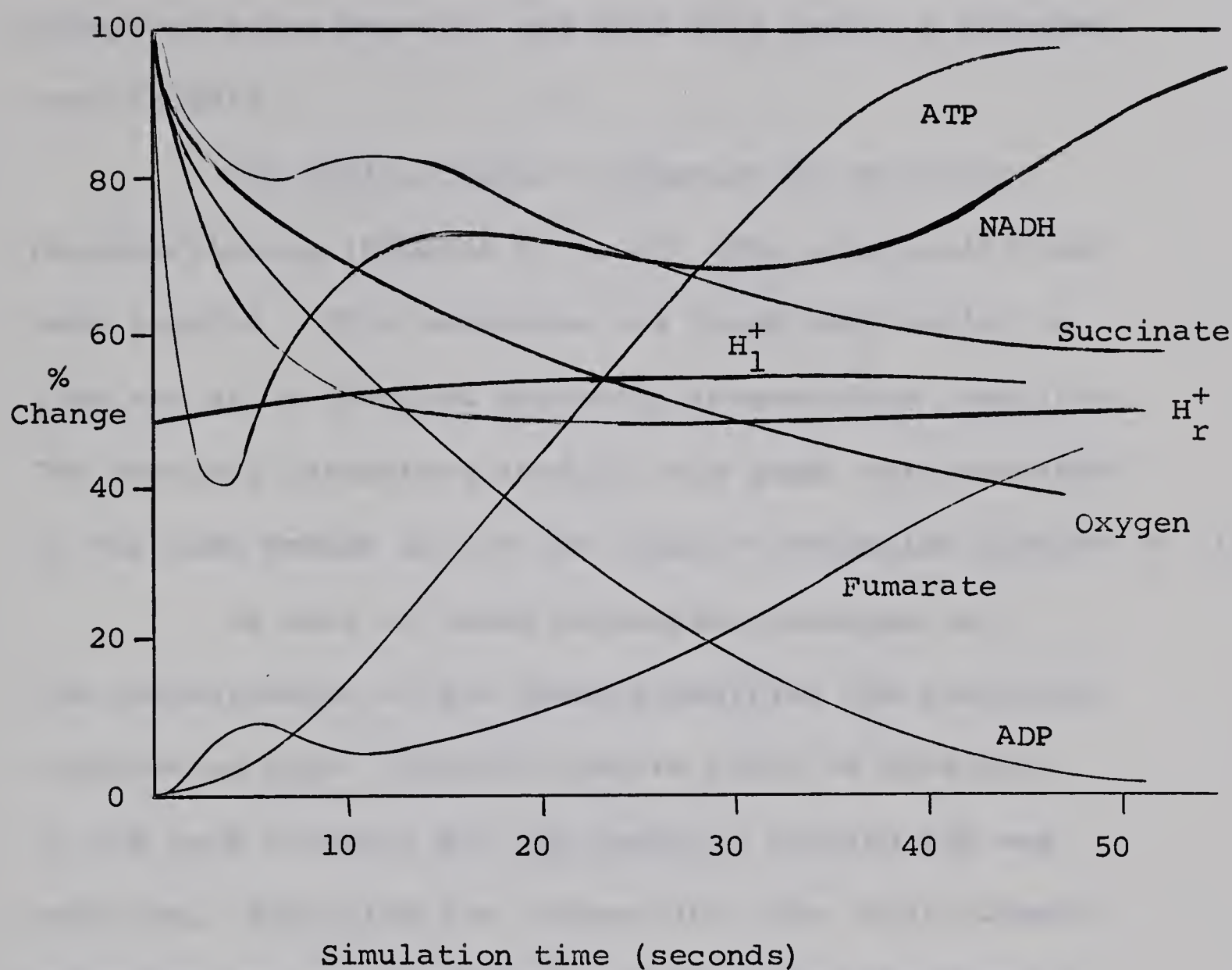


Figure 59. Change in concentration of components of the "Slater" mechanism of oxidative phosphorylation. The initial concentration of ADP in this test was 5.0  $\mu\text{molar}$ .





such a gradient, which is immediately collapsed by the phosphorylation process, and that this cycle is repeated very rapidly.

The "Multi-Chance" mechanism of oxidative phosphorylation (Figures 60 to 64) gave essentially the same results. This mechanism was found much easier to simulate as it involved primarily irreversible reactions. The physical parameters used in this study were obtained in the same manner as for the "Slater" mechanism (Tables 10, 11).

In both of these mechanisms, changes in the concentration of ADP greatly modified the simulated respiration rate. Similar results could be obtained if the rate constant for the reaction forming ATP was modified. Provision for "uncoupling" the "Multi-Chance" mechanism had been included, and when used displayed a very rapid rate of simulated respiration (Uncoupling action was generated by setting the #5 rate constant to greater than zero.).

Extensive trials with a number of mechanisms incorporating glycolysis and oxidative phosphorylation indicated that a very large amount of computer time would be required for complete solution of the appropriate differential equations. As this amount









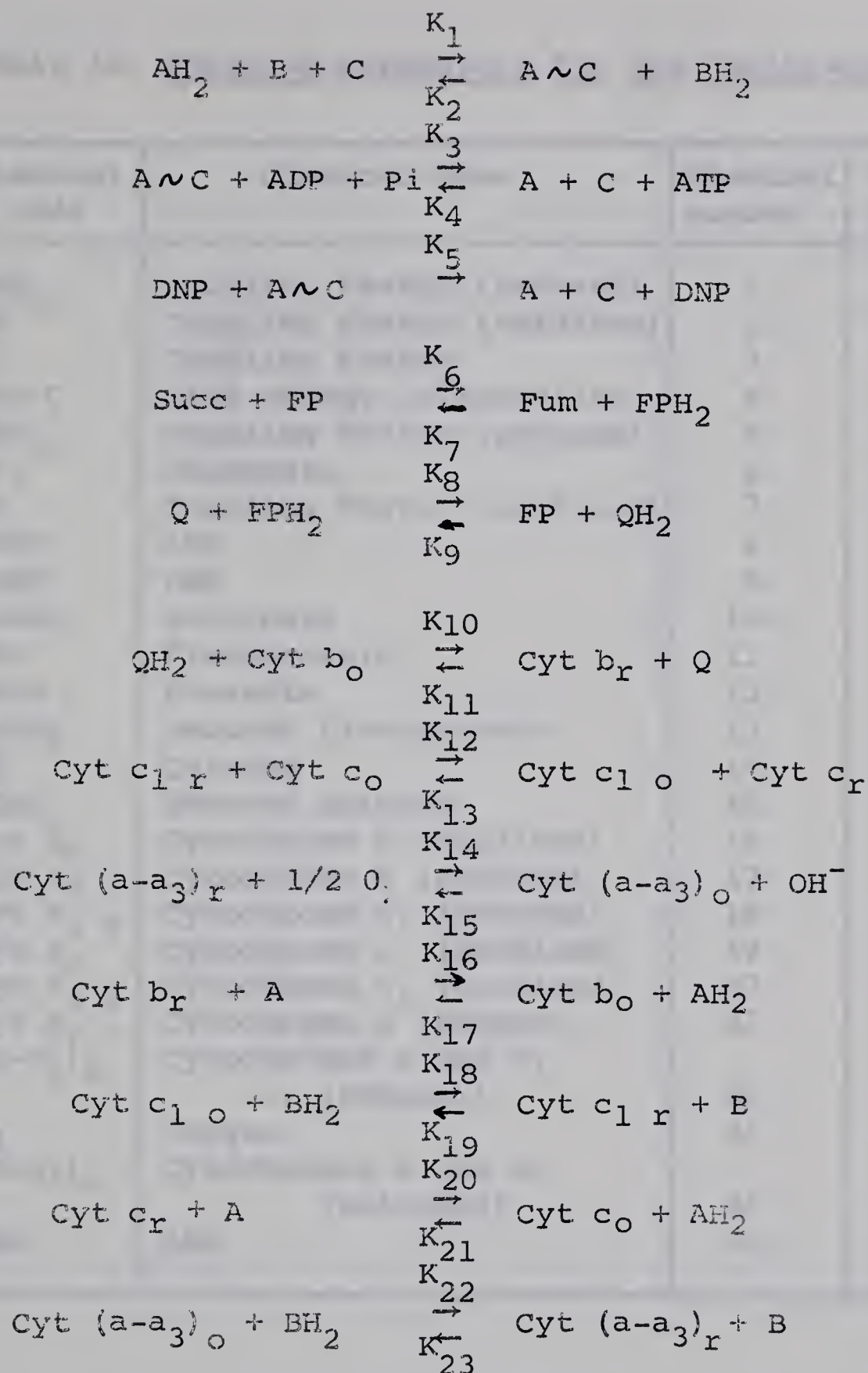


Figure 61. The "Multi-Chance" mechanism of oxidative phosphorylation based on that given by Bonner (1965). Terms are defined in tables 10 and 11.



Table 10. Physical parameters for the "Multi-Chance" mechanism

Chemical code	Chemical name	Chemical number	Concentration (μmoles)
AH <sub>2</sub>	Coupling factor (reduced)	1	10.0
B	Coupling factor (oxidized)	2	10.0
C	Coupling factor	3	1.0
A~C	High energy intermediate	4	0.0
BH <sub>2</sub>	Coupling factor (reduced)	5	10.0
Pi	Phosphate	6	100.0
A	Coupling factor (oxidized)	7	10.0
ATP	ATP	8	0.0
DNP	DNP	9	0.0
Succ	Succinate	10	100.0
FP	Flavoprotein	11	10.0
Fum	Fumarate	12	0.0
FPH <sub>2</sub>	Reduced flavoprotein	13	0.0
Q	Quinone	14	100.0
QH <sub>2</sub>	Reduced quinone	15	0.0
Cyt b <sub>o</sub>	Cytochrome b (oxidized)	16	10.0
Cyt b <sub>r</sub>	Cytochrome b (reduced)	17	0.0
Cyt c <sub>1</sub> r	Cytochrome c <sub>1</sub> (reduced)	18	0.0
Cyt c <sub>o</sub>	Cytochrome c (oxidized)	19	10.0
Cyt c <sub>1</sub> o	Cytochrome c <sub>1</sub> (oxidized)	20	10.0
Cyt c <sub>r</sub>	Cytochrome c (reduced)	21	0.0
Cyt(a-a <sub>3</sub> ) <sub>r</sub>	Cytochromes a and a <sub>3</sub> (reduced)	22	0.0
O <sub>2</sub>	Oxygen	23	240.0
Cyt(a-a <sub>3</sub> ) <sub>o</sub>	Cytochromes a and a <sub>3</sub> (oxidized)	24	10.0
ADP	ADP	25	10.0

Concentrations of the components of this mechanism are given as approximate concentrations before the reaction has commenced.





Table 11. Rate constants for the "Multi-Chance" mechanism

Rate constant $K$ ( )	Value of rate constant (mmoles per second)
1	1000
2	10
3	100
4	1000
5	100
6	1000
7	100
8	1000
9	100
10	1000
11	10
12	1000
13	10
14	1000
15	10
16	100
17	10
18	100
19	10
20	100
21	10
22	100
23	10



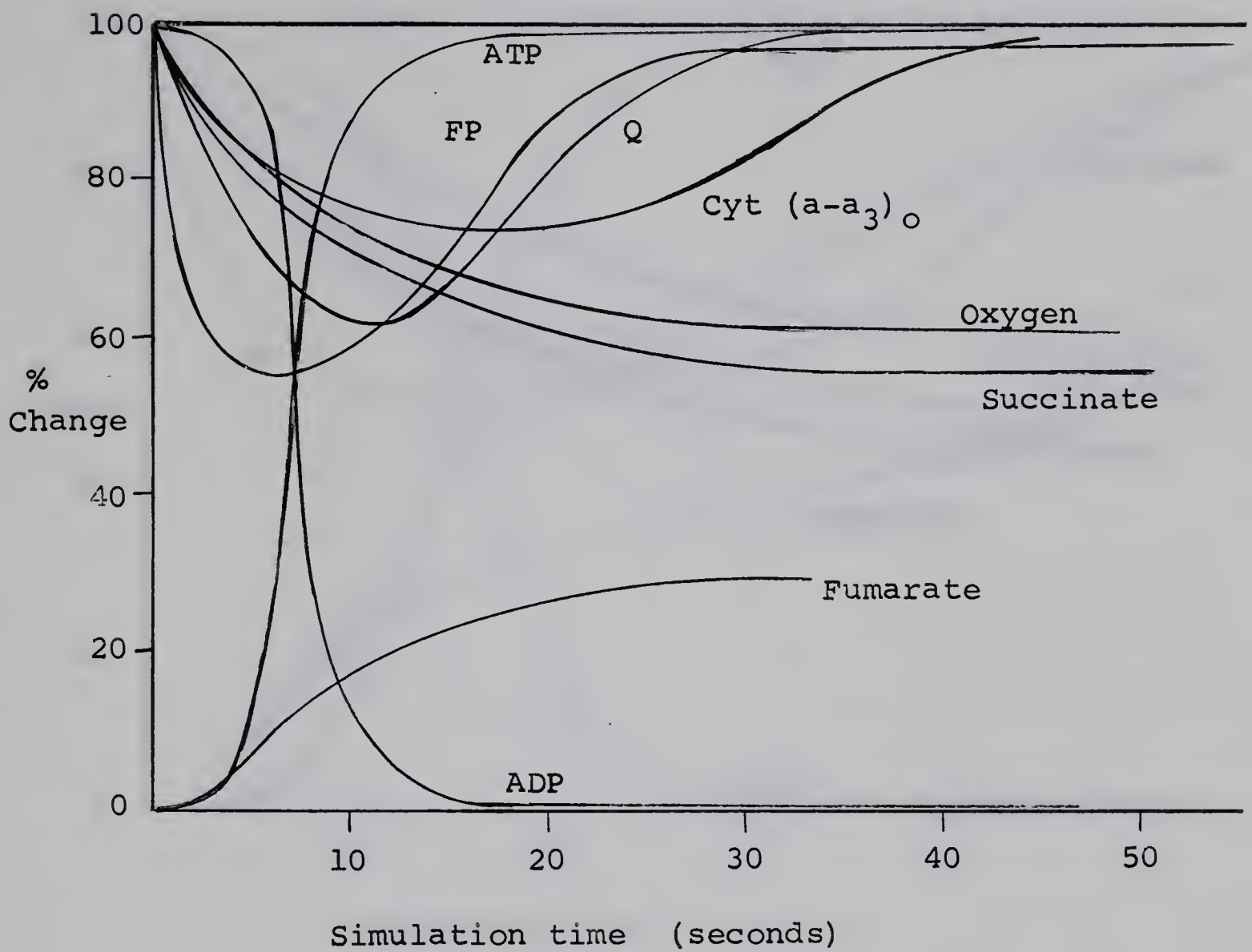


Figure 63. Change in concentration of components of the "Multi-Chance" mechanism of oxidative phosphorylation. The initial concentration of ADP in this system was 0.1  $\mu$ molar.





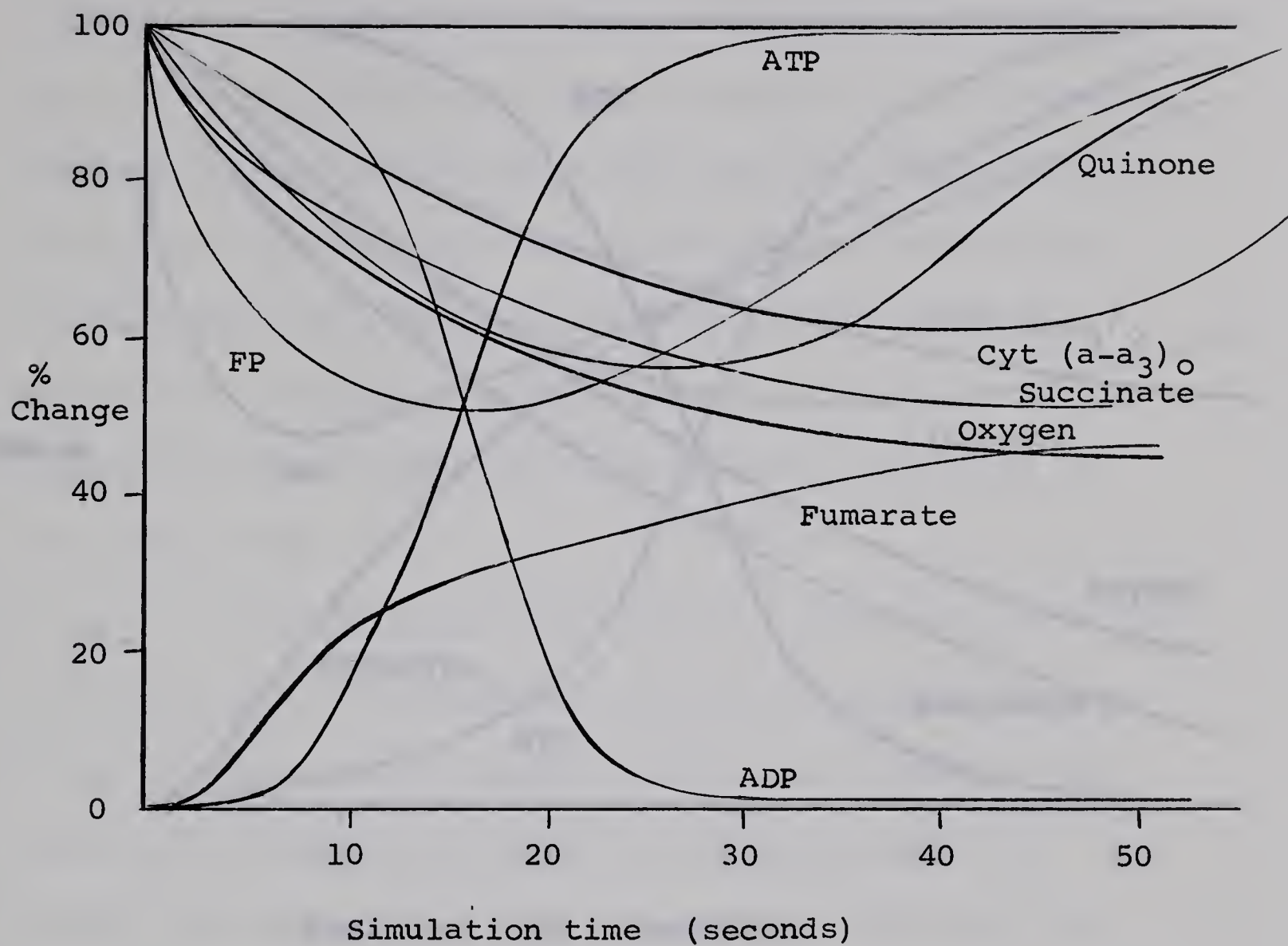


Figure 63. Changes in concentration of components of the "Multi-Chance mechanism of oxidative phosphorylation. The initial concentration of ADP in this test was set at 1.0  $\mu$ molar.



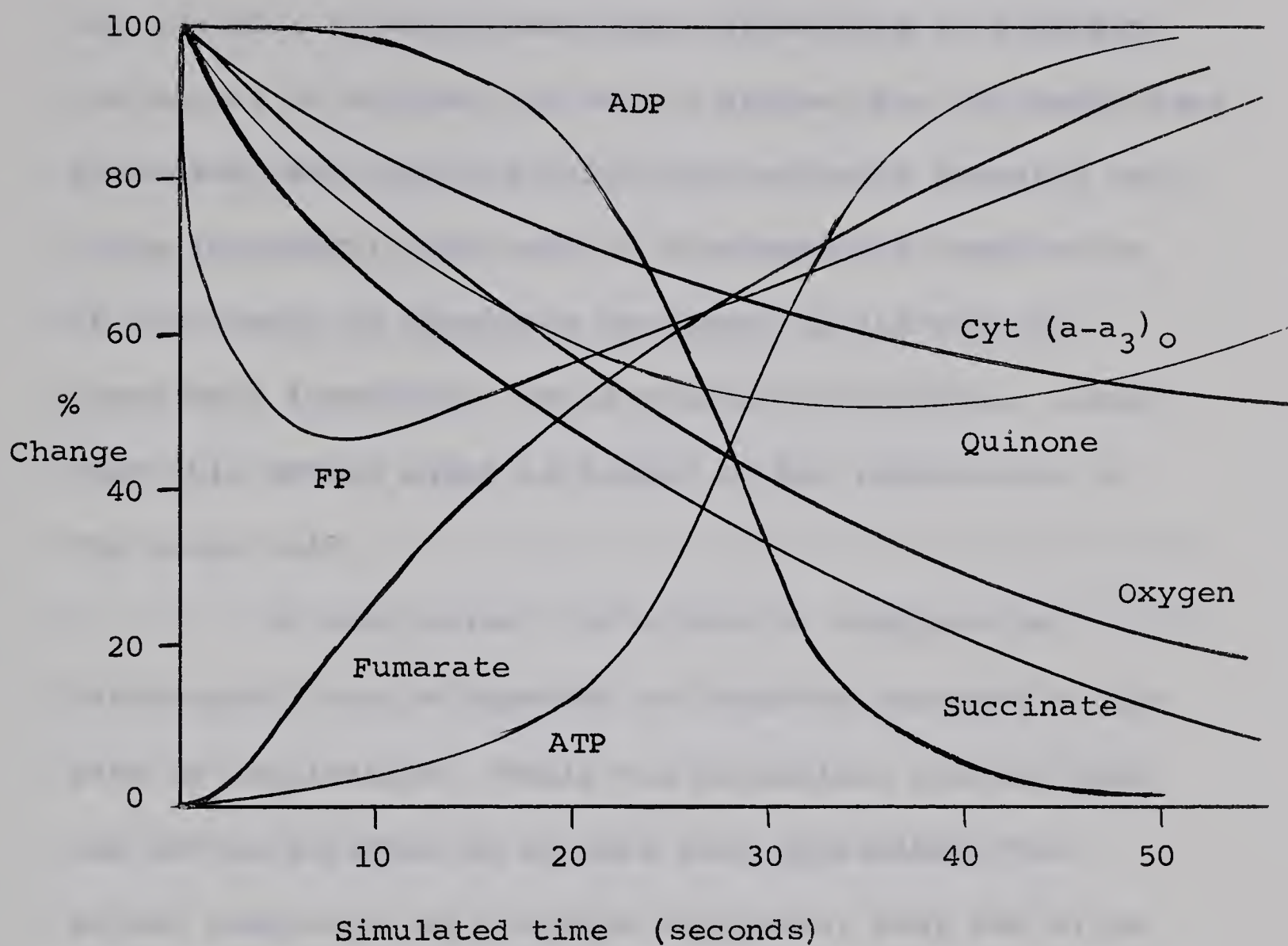


Figure 64. Changes in concentration of components of the "Multi-Chance" mechanism of oxidative phosphorylation. The concentration of ADP in this test was 10  $\mu$ molar.





of computer time was not available, such mechanisms were not tested. It would have been interesting to determine the effect of ethylene on such a system, for the mechanisms presented here indicate only that one could expect a very large increase in the rate of mitochondrial respiration if the amount of phosphate acceptor, or its rate of usage were increased. It is possible, though not likely, that this effect might be hidden by the respiration of the whole cell.

In conclusion, the effect of ethylene on mitochondria can be expected to cause an increase in the rate of respiration. While the mechanisms studied here can not be expected to do more than approximate the actual reactions, or reaction sequences, they can allow one to determine that such an overall change in the rate of respiration of mitochondria would occur if ethylene modifies ADP or ATP utilization, and possibly the ATPase reaction in some manner. This study also points out the usefulness of computers in biochemistry, for the simulation, and testing of models that could not otherwise be analyzed.





General discussion

Ageing processes, such as those found in fruit ripening, or cotyledon senescence are necessarily tied very closely to the respiratory mechanisms of the cell. If ethylene increased the activity of the mitochondrial ATPase, this would result in an increase in the amount of available phosphate acceptor, and respiration. Of course, the rate of utilization of ATP would be more important than changes in its absolute concentration. The observed effect of ethylene in vitro, that of increasing the rate of ATP and ADP induced mitochondrial volume changes may also occur in vivo. This effect is explicable in terms of greater ATPase activity in the presence of ethylene, for an increased concentration of ADP would increase the rate of phosphorylation, and thus cause a rapid decrease in mitochondrial size. (ADP and substrate must be supplied if phosphorylation is to occur.) However, there may be a balance between these two effects.

The inhibition of the ethylene effects on bean cotyledon mitochondria by ouabain is not readily explainable.

ATPase activity usually implies partial uncoupling



of the mitochondrial oxidative processes from mitochondrial phosphorylation, hence one would expect that the increased supply of phosphate acceptor would be used primarily by enzymes in the soluble phase of the cell, thus driving any number of reactions.

It is possible that mitochondrial ATPase and phosphorylative activity may reach a balance, for





as Racker (1965) has suggested, and as Mitchell's theory (Mitchell 1966a) requires, the ATPase activity is only part of the enzyme system involved in phosphorylation, then there must be an obligatory bond between the rate of ATP hydrolysis, and phosphorylation.

Simulation of possible mechanisms of oxidative phosphorylation has indicated that little change in the concentration of ADP is required for a major change in the respiratory behavior of the mitochondrion. Ethylene, at physiological concentrations, may have just enough effect to modify this fine balance between the ATPase system, and the phosphorylation mechanism. The result on cellular metabolism, which is primarily controlled by the rate of mitochondrial metabolism (Chance 1961) would be obvious, for with the increased concentration of phosphate acceptor, glycolysis and other mechanisms would tend to speed up. The carbon dioxide production would increase as well, and one would observe a situation similar to the climateric rise shown by ripening fruit (Spencer 1965), and senescing cotyledons (Spencer, and Olson 1965). It is unfortunate that the effect of oxidative phosphorylation on the glycolytic sequence could not be simulated, for this may have indicated quantitatively the metabolic changes that would occur during this



increase in respiration.

Sacher (1962) has suggested that ethylene affects membrane systems in general. If so, the interactions resulting from an overall change in the rate of transport through membranes would be so great as to cause a general change in the rate of respiration as substrates become immediately available and as inhibitions are modified.

Mitochondria would likely be the first system to respond to ethylene treatment as it exists on a very fine balance. As well, the mitochondrion is one site of ethylene production (Meheriuk 1965). Ethylene may accumulate, presumably in the mitochondrial lipids, until a sufficient concentration is present to cause a major change in mitochondrial characteristics.

This is not to say that general permeability changes could not be induced by ethylene. However, permeability changes could result from the change in the rate of mitochondrial respiration. For instance, yeast grown in continuous culture have thinner cell walls, and a more rapid respiration rate (Korn, and Northcote 1960) than those grown in stationary culture. The increased rate of respiration may reduce the amount of material





available for membrane production, or may even result in a partial degradation of membranes already in existence. Such changes could also occur in senescing cotyledons and in ripening fruit.

The effect of ethylene on rat liver mitochondria is very interesting in that it suggests a long term ageing mechanism in animals, for animal mitochondria also produce ethylene (Chandra, and Spencer 1963a, Gibson 1963).

The actual site of ethylene action is suggested in studies on anaesthetic agents. Pauling (1961) proposed that anaesthetics, such as ethylene, form hydrates which are incorporated into the water structure around protein side chains. If hydrate crystals were to act in this manner, the hydrate would be expected to interfere with ion and electron movement in the protein system, and possibly to block active centres. McMullan, and Jeffrey (1959) found that hydrates of alkylammonium salts, which resemble the lysyl side chains of proteins, could incorporate substances such as xenon that have the same hydrate structure as most anaesthetics (hexakaidecahedron, composed of 12 dodecahedra, each contributing one pentagonal face to the cavity). Miller (1961), and Pauling (1961) indicated that a good





correlation is present between the partial pressure of the anaesthetic agent required to maintain anaesthesia, and the dissociation pressures of the hydrate at  $0^{\circ}\text{C}$ . This held true for a number of anaesthetics, including ethylene.

Because most of the the hydrates formed by anaesthetics are unstable at  $37^{\circ}\text{C}$ ., Pauling suggested that the hydrate formed about the protein side chain would stabilize the hydrate formed by the anaesthetic. Lowering the temperature has been shown to increase the potency of anaesthetics, presumably by allowing for a greater stabilization of the hydrate formed by the anaesthetic in solution (Cherkin, and Catchpool 1964).

The fairly high solubility of ethylene in lipids may also be a factor in the ethylene response, for the very thin protein-phospholipid bilayer membrane of the mitochondrion, in taking up ethylene, could expand a sufficient amount to modify reactions dependent on the membrane thickness (reactions involving transfer of a substrate across the membrane, etcetera).

Thus further research into the mechanism of ethylene action should lead into studies of the effect of ethylene on membrane, and phospholipid conformations,



on possible hydrate-protein interactions, as well as on other biological membranes. Computer simulation of the effects of ethylene on extra-mitochondrial systems would also give further evidence on ethylene's role in cellular metabolism, and in senescence.





## CONCLUSIONS

Mitochondria that were active, and fairly tightly coupled were prepared from a variety of tissues (yeast, rat liver, bean cotyledons, and potato tubers). The mitochondrial fractions were free from debris, and responded in a normal manner to a number of agents capable of causing mitochondrial volume changes.

Ethylene treatment of bean cotyledon, rat liver, and yeast mitochondria increased the rates of mitochondrial volume change induced by ADP and ATP. It was shown that ethylene treatment increased the rate of ATP hydrolysis. Thus an effect on mitochondrial ATPase was sought. Partially purified ATPase preparations from bean cotyledon, and rat liver mitochondria showed no detectable changes in activity in the presence of ethylene as compared to its absence. However, in vivo, ethylene may affect mitochondrial ATPase by virtue of its concentration in the lipids of the membrane as a result of its high solubility in these materials. Its presence could cause changes in the mitochondria membrane conformation or structure by alteration of the arrangement of the fatty acid chains



chains in the phospholipids with respect to each other, and/or by disruption of hydrophobic bonding in proteins of the membrane, possibly including the ATPase protein.

Computer simulation studies with several mechanisms of oxidative phosphorylation indicated that even a slight change in the concentration of ADP, such as that resulting from an increased activity of mitochondrial ATPase, would result in a major change in the pattern of mitochondrial respiration.





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## APPENDIX

### Preparation of graphs

Graphs based on instrument recordings were obtained by photographing the recording, and using a photographic enlarger to obtain an image of the proper size for use in the thesis. These images were carefully traced directly onto the Multilith masters used in the thesis duplication process.

### Preparation of programs for inclusion in thesis

A program was written which accepted these programs as data, and truncated them to fit a 60 space line. As a result, care must be taken when implementing these programs to check for errors resulting from this truncation. As well, all dollar signs in column 1 have been removed to expedite the acceptance of these programs as data.





## Nomenclature of Enzymes

Trivial name	Number	Systematic name
Lactate dehydrogenase	1.1.1.27	L-Lactate:NAD oxidoreductase
Malate dehydrogenase	1.1.1.38	L-Malate:NAD oxidoreductase (decarboxylating)
Glucose oxidase	1.1.3.4.	$\beta$ -D-Glucose: $O_2$ oxido- reductase
Succinate dehydrogenase	1.3.99.1.	Succinate:(acceptor) oxidoreductase
Cytochrome oxidase	1.9.3.1.	Cytochrome c: $O_2$ oxidoreductase
Catalase	1.11.1.6.	$H_2O_2$ : $H_2O_2$ oxidoreductase
Peroxidase	1.11.1.7.	Donor: $H_2O_2$ oxidoreductase
Polyphenol oxidase	1.10.3.1.	Diphenol: $O_2$ oxidoreductase
Phosphofructokinase	2.7.1.11.	ATP:D-fructose-6-phosphate phosphotransferase
Pyruvate kinase	2.7.1.40.	ATP:Pyruvate phospho- transferase
Cellulase	3.2.1.4.	$\beta$ -1,4-Glucan 4-glucano- hydrolase
ATPase	3.6.1.4.	ATP phosphohydrolase
Carboxylase	4.1.1.1.	2-oxo-acid carboxy-lyase
Aldolase	4.1.2.7.	Ketose-1-phosphate aldehyde lyase
Pyruvate carboxylase	6.4.1.1.	Pyruvate: $CO_2$ ligase (ADP)
Glutathione peroxidase	not given	not given



## APPENDIX

"Regular simulator" program (IBM 7040)





JOB 765003 SINGLE ENZYME

TIME 15,3000

IBJOB MAP

IBFTC TC

COMMON F,XK,Y,D,X,DL,XK1,XK2,XK3,XK4,XK5,TL,T,XLP,E,  
1ERROR,H,DT,KC,N,J,M,KQ,KP,KS,KBLANK,GI,L,LT,LS,  
2LR,LQ,LO,LP,LM,LN,LL,YA,YM,KSTEP,KSTOP,KDUMP,  
3KRUN,KDAMP,KNT,W,AR,BR,CR,KRKG,KM,KN,KR,KT,P,U,  
4KB,K,R,LA,LB,LC,LD,LE,LF,LG,LH,LI,LJ,LK

DIMENSION F(100),XK(100),Y(100),D(100),X(10),DL(25),  
1XK1(100),XK3(100),XK4(100),XK5(100),KS(10,3),YA(100),  
2YM(100),AR(4),BR(4),CR(4),W(100),Z(100),P(100),  
3U(100),R(100),KA(98)

89 FORMAT(1X,E10.3,5X,I5)

90 FORMAT(1X, 5H CLOB)

93 FORMAT(1X,26H PROGRAM HAS ENTERED RUNGE)

94 FORMAT(1X,18H NUMBER OF CYCLES=, I5)

C

C CONSTANTS IN THE RUNGE-KUTTE-GILL SUBROUTINE

C

AR(1)=0.5

AR(2)=0.2929

AR(3)=1.7071

AR(4)=0.1667

BR(1)=2.0

BR(2)=1.0

BR(3)=1.0

BR(4)=2.0

CR(1)=0.5

CR(2)=0.2929

CR(3)=1.7071

CR(4)=0.5

L=0

KRKG=0

KSTEP=0

KRUN=0

KDUMP=0

LL=0

LM=0

LN=0

LO=0

LP=0

LQ=0

LR=0

LS=0

LT=0

LA=0

LB=0

LD=0

LE=0

LC=0

C LT=TC

C LQ=READB

C LM=CLOB

C LP=GRAPH



```

C      LL=INTEG
C      LO=SOLVE
C      LR=RAWD
C      LN=EVALV
C      LS=DERV
C      LA=RUNGE
C      LB=KATA
C      LD=NORMAL
C      LE=CCNSV
C
C      READS IN DATA
C
36    CALL READB
C
C      IF KM IS LESS THAN 1, CALLS FPTSW
C
      IF(KM.LT.1) GO TO 300
      GO TO 301
300   CALL FPTSW
C
C      TURNS OFF ERROR MESSAGE RESULTING FROM
C      AC, MQ OVERFLOW
301   CONTINUE
C
C      NORMALIZING CALLED FOR IF KB IS GT ONE
C
      IF(KB.LT.1) GO TO 14
      CALL NORMAL
C
C      CALLS DERIVATIVES
C
14    CALL DERV
C
C      OUTPUTS MATERIAL READ IN
C
      CALL RAWD
C
C      ZERO'S GRAPH
C
      CALL GRAPH
      LT=LT+1
C
C      CALLS RUNGE ROUTINE IF KNT IS LESS THAN 2
C
      IF(KNT-2) 18,15,15
C
C      CALLS RUNGE KUTTA MERSON INTEGRATION PROCEDURE
C
15    CALL INTEG
      CALL DERV
C
C      CALLS CLOBBER OR GRAPH ROUTINES
C
      IF(KDAMP-2) 16,17,17
17    CALL GRAPH

```





```

      L=L
C
C   SETS MINIMUM DT ALLOWED
C
      IF(DT.LT.0.1000E-15) GO TO 35
      IF(T-TL) 20,30,30
C
C   CHECKS TO FIND OUT WHETHER ALL DERIVATIVES
C   ARE IN RANGE
C
20   DO 88 I=1,N
      U(I)=ABS(D(I))
      IF(U(I).LT.0.100E-25) GO TO 30
      IF(U(I).GT.0.100E+25) GO TO 30
88   CONTINUE
C
C   FIRST OUTPUT
C
      IF(T-DL(L)) 15,15,10
10   CALL RAWD
      DL(L)=2.*TL
      GO TO 15
C
C   ENTERS RUNGE KUTTA GILL ROUTINE
C
18   CALL RUNGE
      IF(KRKG.LT.1) GO TO 24
      WRITE(6,93)
      KRKG=KRKG+1
C
C   CHECKS WHETHER CLOBBER ROUTINE IS TO BE USED
C
24   CONTINUE
      IF(KRUN-2) 19,21,21
19   WRITE(6,90)
      KRUN=KRUN+1
      GO TO 21
C
C   CHOICE BETWEEN GRAPH OR CLOBBER ROUTINE
C
21   CALL DERV
      IF(KDAMP-2) 27,28,28
C
C   CALLS GRAPH ROUTINE AND INCREMENTS TIME
C
28   CALL GRAPH
      T=T+DT
      IF(DT.LT.0.1000E-25) GO TO 30
      IF(T-TL) 23,30,30
C
C   CHECKS LIMITS
C
23   DO 91 I=1,N
      U(I)=ABS(D(I))
      IF(U(I).LT.0.100E-25) GO TO 30

```



```

      IF(U(I).GT.0.1COE+25) GO TO 30
C
91  CONTINUE
C
C   RETURN AFTER CALCULATING THE NEW DERIVATIVE
C   TO RUNGE
C   GO TO 18
C
C   IF DT VALUES EXCEED LIMITS,DECREASES ERROR CRITERIA
C   AND STARTS CALCULATION OVER AGAIN
C
35  ERROR=0.5*ERROR
    KDUMP=KDUMP+1
    GO TO 36
C
C   CHECKS WHETHER DERIVATIVES ARE TOO LOW, IF
C   SO, OUTPUTS AND ENDS
C
30  WRITE(6,89) U(I),I
    CALL RAWD
    GO TO 31
16  CONTINUE
C
C   FOR NON GRAPH ROUTINE, CALLS CLOBBER AND ASKS
C   FOR A GIVEN NO OF OUTPUTS
C
    CALL CLOB
    KSTEP=KSTEP+1
    IF(KSTEP-KSTOP) 15,15,31
27  CONTINUE
    CALL CLOB
    KSTEP=KSTEP+1
    IF(KSTEP-KSTOP) 19,19,31
31  WRITE(6,94) DSTEP
    END

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IBFTC READB

C  
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C  
C

THIS PROGRAM READS IN ALL DATA ON DATA CARDS

SUBROUTINE READB

COMMON F,XK,Y,D,X,DL,XK1,XK2,XK3,XK4,XK5,TL,T,XLP,E,  
1ERROR,H,DT,KC,N,J,M,KQ,KP,KS,KBLANK,GI,L,LT,LS,  
2LR,LQ,LO,LP,LM,LN,LL,YA,YM,KSTEP,KSTOP,KDUMP,  
3KRUN,KDAMP,KNT,W,AR,BR,CR,KRKG,KM,KN,KR,KT,P,U,  
4KB,K,R,LA,LB,LC,LD,LE,LF,LG,LH,LI,LJ,LK  
DIMENSION F(100),XK(100),Y(100),D(100),X(10),DL(25),  
1XK1(100),XK3(100),XK4(100),XK5(100),KS(10,3),YA(100),  
2YM(100),AR(4),BR(4),CR(4),W(100),Z(100),P(100),  
3U(100),R(100),KA(98)

LQ=LQ+1

300 FORMAT(5I5,A1)

301 FORMAT(7E10.4)

302 FORMAT(19HOVELOCITY CONSTANTS//(7E16.4//))

303 FORMAT(21HOINITIAL DELTA TIME = , E12.4/13H TIME LIM

1IT = ,E12.4/

1 8H ERROR =,E12.4)

304 FORMAT(13HOSCALE VALUES//(7E16.4//))

305 FORMAT(2I5,1X,A1)

306 FORMAT(1X,18H NO OF CHEMICALS =, I5)

307 FORMAT(2H Y,I5,7H SCALE,I5,8H SYMBOL,2X,A2)

308 FORMAT(15HOCONCENTRATIONS//(7E16.4//))

309 FORMAT(1X,34H MAXIMUM OR MINIMUM CONCENTRATIONS//(7E16  
1.4//))

310 FORMAT(//1X)

311 FORMAT(4I5)

312 FORMAT(1X,19H SUBROUTINE CLOBBER,5X,7HKDAMP =, I5,5X,1  
16H NUMBER OF  
1DUMPS=, I5)

313 FORMAT(1X,42H NORMALIZING CALLED FOR IF KB IS GE 1 KB  
1=, I5)

314 FORMAT(1X,38H INTEGRATION PROCEDURE=(RKG=1)(RKM=2)', I  
12)

315 FORMAT(1X,26H TAPE CALLED FOR IF KT = 2,I5)

317 FORMAT(1X,36H IF KM IS LESS THAN 1 CALL FPTSW =, I5  
1)

318 FORMAT(1X,27H NO OF VELOCITY CONSTANTS =, I5)

319 FORMAT(1X,31H NO OF SCALE VALUES FOR GRAPH =, I5)

320 FORMAT(1X,22H NO OF GRAPH OUTPUTS =, I5)

321 FORMAT(1X,29H NO OF INTERMEDIATE OUTPUTS =, I5)

322 FORMAT(I5,5X,2E10.3)

323 FORMAT(1X,25H CHEMICAL TO BE CHANGED =, I5)

324 FORMAT(1X,43H CONCENTRATION WHERE CHANGE IS TO BE MADE  
1 =, E10.3)

325 FORMAT(1X,21H AMOUNT TO BE ADDED =, E10.3)

READ(5,300) N,J,M,KP,KQ,KBLANK

WRITE(6,306) N

WRITE(6,318) J

WRITE(6,319) M



```

WRITE(6,320) KP
WRITE(6,321) KG
READ(5,301) (YA(I),I=1,N)
DO 11 I=1,N
11 Y(I)=1.000*YA(I)
READ(5,301) (XK(I),I=1,J)
READ(5,301) DT,TL,ERROR
WRITE(6,303) DT,TL,ERROR
READ(5,301) (X(I),I=1,M)
READ(5,305) ((KS(I,IJ),IJ=1,3),I=1,KP)
READ(5,311) KDAMP,KSTOP
WRITE(6,312) KDAMP,KSTOP

C
C KDAMP = 1, CALLS CLOBBBER
C KDAMP = 2, GOES THRU NORMAL ROUTINE
C

READ(5,311) KNT,KM,KT,KB
READ(5,322) KYI,YAMT,YMT
WRITE(6,314) KNT

C
C INTEGRATION PROCEDURE CHOICE
C IF KNT EQUALS ONE, CALLS RKG
C IF KNT EQUALS TWO, CALLS RKM
C

WRITE(6,315) KT

C
C KT INDICATES WHETHER TWO OUTPUT PROGRAM TO BE CALLED
C IF KT EQUALS 2 THEN CALLS THIS PROGRAM
C

WRITE(6,317) KB

C
C IF KB IS GREATER THAN ONE, NORMALIZING IS CALLED FOR.
C

WRITE(6,313) KM

C
C IF KM IS GREATER THAN TWO, MUST READ IN MAXIMUM VALUES
C FOR CHEMICALS
C IF KM IS LESS THAN ONE, MUST CALL FPTSW
C

WRITE(6,323) KYI
WRITE(6,324) YAMT
WRITE(6,325) YMT

C
C KYI EQUALS CHEMICAL TO BE CHANGED
C KYAMT EQUALS CONCENTRATION AT WHICH CHANGE IS TO BE
C MADE
C KAMT EQUALS THE NEW CONCENTRATION
C

WRITE(6,308) (Y(I),I=1,N)
WRITE(6,302) (XK(I),I=1,J)
WRITE(6,310)
WRITE(6,307) ((KS(I,IJ),IJ=1,3),I=1,KP)
WRITE(6,310)
WRITE(6,304) (X(I),I=1,M)
WRITE(6,310)

```







```

100 IF(KM.GT.2) GO TO 12
    GO TO 13
12  READ(5,301) (P(I),I=1,N)
    WRITE(6,309)(P(I),I=1,N)
13  CONTINUE
    IF(KQ) 10,5,10
10  READ(5,301) (DL(I),I=1,KQ)
    GO TO 6
C
C    IF DL IS NOT SET, IT ENTERS BY ITSELF
C
5   DL(1)=2.*TL
    L=1
C
C    SETS LIMITS FOR GRAPH OUTPUT AND ERROR LEVEL
C
6   ERROR=.5*ERROR
    XLP=0.
    GI=TL/100.
    TL=TL*.99
    RETURN
    END

```



IBFTC KATA

C  
C  
C  
C  
C  
C

THIS PROGRAM ALLOWS FOR SIMULTANEOUS WRITING ON  
TAPE DRIVES 6 + 4

SUBROUTINE KATA

COMMON F,XK,Y,D,X,DL,XK1,XK2,XK3,XK4,XK5,TL,T,XLP,E,  
1ERROR,H,DT,KC,N,J,M,KQ,KP,KS,KBLANK,GI,L,LT,LS,  
2LR,LQ,LO,LP,LM,LN,LL,YA,YM,KSTEP,KSTOP,KDUMP,  
3KRUN,KDAMP,KNT,W,AR,BR,CR,KRKG,KM,KN,KR,KT,P,U,  
4KB,K,R,LA,LB,LC,LD,LE,LF,LG,LH,LI,LJ,LK  
DIMENSION F(100),XK(100),Y(100),D(100),X(10),DL(25),  
1XK1(100),XK3(100),XK4(100),XK5(100),KS(10,3),YA(100),  
2YM(100),AR(4),BR(4),CR(4),W(100),Z(100),P(100),  
3U(100),R(100),KA(98)

LB=LB+1

301 FORMAT(12E10.3)

302 FORMAT(1X,15H CONCENTRATIONS)

303 FORMAT(1X,7H FLUXES)

304 FORMAT(1X,12H DERIVATIVES)

305 FORMAT(1X,7H DT = ,E10.3,9H TIME = , E10.3)

WRITE(4,302)

WRITE(4,301) (Y(I),I=1,N)

WRITE(4,303)

WRITE(4,301) (F(I),I=1,J)

WRITE(4,304)

WRITE(4,301) (D(I),I=1,N)

WRITE(4,305) DT,T

RETURN

END





IBFTC CLOB

C  
C  
C  
C  
C

THIS ROUTINE IS DESIGNED TO DUMP ALL OUTPUT DIRECTLY

SUBROUTINE CLOB

COMMON F,XK,Y,D,X,DL,XK1,XK2,XK3,XK4,XK5,TL,T,XLP,E,  
1ERROR,H,DT,KC,N,J,M,KQ,KP,KS,KBLANK,GI,L,LT,LS,  
2LR,LQ,LO,LP,LM,LN,LL,YA,YM,KSTEP,KSTOP,KDUMP,  
3KRUN,KDAMP,KNT,W,AR,BR,CR,KRKG,KM,KN,KR,KT,P,U,  
4KB,K,R,LA,LB,LC,LD,LE,LF,LG,LH,LI,LJ,LK

DIMENSION F(100),XK(100),Y(100),D(100),X(10),DL(25),  
1XK1(100),XK3(100),XK4(100),XK5(100),KS(10,3),YA(100),  
2YM(100),AR(4),BR(4),CR(4),W(100),Z(100),P(100),  
3U(100),R(100),KA(98)

300 FORMAT(1X,12HODERIVATIVES)

301 FORMAT(1X,10(2X,E10.3))

302 FORMAT(1X,7HOFUXES)

303 FORMAT(1X,15HOCNCENTRATIONS)

306 FORMAT(1X,36H PROGRAM HAS ENTERED CLOBBER ROUTINE)

LM=LM+1

C  
C  
C

COUNTER ROUTINE TO INDICATE ENTRY INTO THE SUBROUTINE

IF(KR-1) 304,305,305

304 KR=KR+1

WRITE(6,306)

GO TO 305

305 WRITE(6,300)

WRITE(6,301) (D(I),I=1,N)

WRITE(6,302)

WRITE(6,301) (F(I),I=1,J)

WRITE(6,303)

WRITE(6,301) (Y(I),I=1,N)

RETURN

END



IBFTC RUNGE

```
C
C
C   RUNGE KUTTA GILL AS PROPOSED BY S. GILL 'A PROCESS FOR
1  THE STEP
C   BY STEP INTEGRATION OF DIFFERENTIAL EQUATIONS ON
C   AN AUTOMATIC DIGITAL COMPUTING MACHINE'
C   PROC.CAMBRIDGE PHILOS. SOC., 47, 96-108 (1951)
C   THIS IS A FOURTH ORDER PROCEDURE
C
C   SUBROUTINE RUNGE
      COMMON F,XK,Y,D,X,DL,XK1,XK2,XK3,XK4,XK5,TL,T,XLP,E,
1  IERROR,H,DT,KC,N,J,M,KQ,KP,KS,KBLANK,GI,L,LT,LS,
      2LR,LQ,LO,LP,LM,LN,LL,YA,YM,KSTEP,KSTOP,KDUMP,
      3KRUN,KDAMP,KNT,W,AR,BR,CR,KRKG,KM,KN,KR,KT,P,U,
      4KB,K,R,LA,LB,LC,LD,LE,LF,LG,LH,LI,LJ,LK
      DIMENSION F(100),XK(100),Y(100),D(100),X(10),DL(25),
      1XK1(100),XK3(100),XK4(100),XK5(100),KS(10,3),YA(100),
      2YM(100),AR(4),BR(4),CR(4),W(100),Z(100),P(100),
      3U(100),R(100),KA(98)
      LA=LA+1
      REAL Q(10)
      DO 1 J=1,4
      CALL DERV
      DO 1 I=1,N
      DXX=AR(J)*D(I)-BR(J)*Q(I)
      Y(I)=Y(I)+DT*DXX
1  Q(I)=Q(I)+3.*DXX-CR(4)*D(I)
      RETURN
      END
```





# IBFTC GRAPH

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C

## GRAPH ROUTINE USING DOUBLE MATRIX

### SUBROUTINE GRAPH

COMMON F,XK,Y,D,X,DL,XK1,XK2,XK3,XK4,XK5,TL,T,XLP,E,  
1ERROR,H,DT,KC,N,J,M,KQ,KP,KS,KBLANK,GI,L,LT,LS,  
2LR,LQ,LO,LP,LM,LN,LL,YA,YM,KSTEP,KSTOP,KDUMP,  
3KRUN,KDAMP,KNT,W,AR,BR,CR,KRKG,KM,KN,KR,KT,P,U,  
4KB,K,R,LA,LB,LC,LD,LE,LF,LG,LH,LI,LJ,LK

DIMENSION F(100),XK(100),Y(100),D(100),X(10),DL(25),  
1XK1(100),XK3(100),XK4(100),XK5(100),KS(10,3),YA(100),  
2YM(100),AR(4),BR(4),CR(4),W(100),Z(100),P(100),  
3U(100),R(100),KA(98)

300 FORMAT(F7.2,2H .,98A1,1H.)

LP=LP+1

DO 100 IA=1,98

100 KA(IA)=KBLANK

DO 7 IA=1,KP

KSY=KS(IA,1)

KSX=KS(IA,2)

KC=100.\*Y(KSY)/X(KSX)

IF(KC) 7,7,6

6 IF(KC-98) 110,110,7

110 KA(KC)=KS(IA,3)

7 CONTINUE

5 WRITE(6,300) T,KA

CALL BCLEAR

RETURN

END



IBFTC INTEG

```
C
C
C
C
      SUBROUTINE INTEG
      COMMON F,XK,Y,D,X,DL,XK1,XK2,XK3,XK4,XK5,TL,T,XLP,E,
1ERROR,H,DT,KC,N,J,M,KQ,KP,KS,KBLANK,GI,L,LT,LS,
2LR,LQ,LO,LP,LM,LN,LL,YA,YM,KSTEP,KSTOP,KDUMP,
3KRUN,KDAMP,KNT,W,AR,BR,CR,KRKG,KM,KN,KR,KT,P,U,
4KB,K,R,LA,LB,LC,LD,LE,LF,LG,LH,LI,LJ,LK
      DIMENSION F(100),XK(100),Y(100),D(100),X(10),DL(25),
1XK1(100),XK3(100),XK4(100),XK5(100),KS(10,3),YA(100),
2YM(100),AR(4),BR(4),CR(4),W(100),Z(100),P(100),
3U(100),R(100),KA(98)
      LL=LL+1
C
C      RUNGE-KUTTA-MERSON SOLUTIONS
C
C
C      CHECKS ERROR
C
1      CALL SOLVE
      E=0.
      DO 22 KC=1,N
      H=ABS (XK1(KC)-4.5*XK3(KC)+4.*XK4(KC)-.5*XK5(KC))
      IF(H-E) 22,22,18
18      E=H
22      CONTINUE
      IF(E-ERROR) 10,5,5
5      DT=.5*DT
      GO TO 1
C
C      SETS PRINT OUT INTERVALS
C
10      IF(T+DT-(XLP+GI)) 13,12,12
12      OLDDT=DT
      DT= XLP+GI-T
      CALL SOLVE
      CALL EVALV
      DT=OLDDT
      T=XLP+GI
      XLP=T
      RETURN
C
C      CHECKS ERROR, IF OK, INCREASES DT
C
13      IF(E-ERROR/32.) 14,20,20
14      DT=2.*DT
      GO TO 1
20      CONTINUE
      CALL EVALV
C
C      INCREMENTS T
C
```





```
100 T=T+DT
110 GO TO 1
120 END
```



IBFTC SOLVE

C  
C THIS IS A RUNGE KUTTA MERSON INTEGRATION PROCEDURE  
C AND WAS OBTAINED FROM DR.D. GARFINKEL, JRF, PHILA, 196  
16

C IT IS A FIFTH ORDER PROCEDURE

C  
SUBROUTINE SOLVE  
COMMON F,XK,Y,D,X,DL,XK1,XK2,XK3,XK4,XK5,TL,T,XLP,E,  
1ERROR,H,DT,KC,N,J,M,KQ,KP,KS,KBLANK,GI,L,LT,LS,  
2LR,LQ,LO,LP,LM,LN,LL,YA,YM,KSTEP,KSTCP,KDUMP,  
3KRUN,KDAMP,KNT,W,AR,BR,CR,KRKG,KM,KN,KR,KT,P,U,  
4KB,K,R,LA,LB,LC,LD,LE,LF,LG,LH,LI,LJ,LK  
DIMENSION F(100),XK(100),Y(100),D(100),X(10),DL(25),  
1XK1(100),XK3(100),XK4(100),XK5(100),KS(10,3),YA(100),  
2YM(100),AR(4),BR(4),CR(4),W(100),Z(100),P(100),  
3U(100),R(100),KA(98)  
LC=LO+1  
H=.33333333\*DT  
DO 10 KC=1,N  
XK5(KC)=Y(KC)  
XK1(KC)=H\*D(KC)  
10 Y(KC)=XK5(KC)+XK1(KC)  
CALL DERV  
DO 20 KC=1,N  
XK3(KC)=H\*D(KC)  
20 Y(KC)=XK5(KC)+.5\*(XK1(KC)+ XK3(KC))  
CALL DERV  
DO 30 KC=1,N  
XK3(KC)=H\*D(KC)  
30 Y(KC)=XK5(KC)+.375\*XK1(KC)+ 1.125\*XK3(KC)  
CALL DERV  
DO 40 KC=1,N  
XK4(KC)=H\*D(KC)  
40 Y(KC)=XK5(KC)+1.5\*XK1(KC)-4.5\*XK3(KC) +6.\*XK4(KC)  
CALL DERV  
DO 50 KC=1,N  
Y(KC)=XK5(KC)  
50 XK5(KC)=H\*D(KC)  
CALL DERV  
RETURN  
END





IBFTC RAWD

C  
C  
C  
C

ALLOWS FOR INITIAL AND SECONDARY DUMPS OF INFORMATION

SUBROUTINE RAWD

COMMON F,XK,Y,D,X,DL,XK1,XK2,XK3,XK4,XK5,TL,T,XLP,E,  
1ERROR,H,DT,KC,N,J,M,KQ,KP,KS,KBLANK,GI,L,LT,LS,  
2LR,LQ,LO,LP,LM,LN,LL,YA,YM,KSTEP,KSTOP,KDUMP,  
3KRUN,KDAMP,KNT,W,AR,BR,CR,KRKG,KM,KN,KR,KT,P,U,  
4KB,K,R,LA,LB,LC,LD,LE,LF,LG,LH,LI,LJ,LK  
DIMENSION F(100),XK(100),Y(100),D(100),X(10),DL(25),  
1XK1(100),XK3(100),XK4(100),XK5(100),KS(10,3),YA(100),  
2YM(100),AR(4),BR(4),CR(4),W(100),Z(100),P(100),  
3U(100),R(100),KA(98)

LR=LR+1

16 FORMAT(1HJ, 72H TC DERV READ RAWD SOLV GRAP CLOB EVAL  
1 INTE RUNG

1KATA NORM CONS REREAD)

17 FORMAT(1HJ,15I5)

300 FORMAT(//15H CONCENTRATIONS/)

301 FORMAT(// 15H REACTION RATES/)

302 FORMAT(// 16H REACTION FLUXES/)

303 FORMAT(7E16.8/)

304 FORMAT(/ 5HOTIME,F11.4 //)

305 FORMAT(/ 3HODT,E20.15//)

306 FORMAT(1X,20H CONSERVATION VALUES/)

307 FORMAT(1X,45H SUBSTRATE PLUS PRODUCT ENZYME PLUS COM  
1PLEX)

308 FORMAT(10X,E10.3,15X,E10.3)

WRITE(6,300)

WRITE(6,303) (Y(I),I=1,N)

WRITE(6,301)

WRITE(6,303) (D(I),I=1,N)

WRITE(6,302)

WRITE(6,303) (F(I),I=1,J)

WRITE(6,306)

WRITE(6,307)

CALL CONS

WRITE(6,308) R(1),R(2)

WRITE(6,304) T

WRITE(6,305) DT

WRITE(6,16)

WRITE(6,17) LT,LS,LQ,LR,LO,LP,LM,LN,LL,LA,LB,LD,LE,LC

CALL BCLEAR

IF(KQ) 10,20,10

10 IF(L-KQ) 14, 12,14

12 L=L+1

DL(L)=2.\*TL

RETURN

14 L=L+1

20 RETURN

END



```

IBFTC NORMAL
C
C
C   THIS PROGRAM CALCULATES NORMALIZED
C   CONCENTRATIONS AND RATE VALUES AND PRINTS
C
C   SUBROUTINE NORMAL
C   COMMON F,XK,Y,D,X,DL,XK1,XK2,XK3,XK4,XK5,TL,T,XLP,E,
1ERROR,H,DT,KC,N,J,M,KQ,KP,KS,KBLANK,GI,L,LT,LS,
2LR,LQ,LO,LP,LM,LN,LL,YA,YM,KSTEP,KSTOP,KDUMP,
3KRUN,KDAMP,KNT,W,AR,BR,CR,KRKG,KM,KN,KR,KT,P,U,
4KB,K,R,LA,LB,LC,LD,LE,LF,LG,LH,LI,LJ,LK
C   DIMENSION F(100),XK(100),Y(100),D(100),X(10),DL(25),
1XK1(100),XK3(100),XK4(100),XK5(100),KS(10,3),YA(100),
2YM(100),AR(4),BR(4),CR(4),W(100),Z(100),P(100),
3U(100),R(100),KA(98)
301  FORMAT(1X,26HONORMALIZED CONCENTRATIONS)
302  FORMAT(1X,10(2X,F10.5))
303  FORMAT(1X,26HONORMALIZED RATE CONSTANTS)
304  FORMAT(1X,35H PERCENT OF MAXIMUM CONCENTRATIONS )
LD=LD+1
C
C   MAKES SURE THAT LOG WILL NOT BE LOG OF ZERO
C
278  DO 279 I=1,N
    IF(Y(I).LE.0.) GO TO 282
279  CONTINUE
    GO TO 285
C
C   IF Y(I) = 0 , SETS IN NEW VALUE
C
282  Y(I)=0.100E-20
    GO TO 278
285  CONTINUE
C
C   TAKES LOG VALUE
C
    DO 280 I=1,N
280  Z(I)=ALOG(Y(I))
C
C   WRITES OUT LOG
C
287  WRITE(6,301)
    WRITE(6,302) (Z(I),I=1,N)
C
C   CALCULATES OUT PERCENT OF MAXIMUM VALUE
C
    DO 291 I=1,N
291  Y(I)=Y(I)/P(I)
    WRITE(6,304)
    WRITE(6,302) (Y(I),I=1,N)
290  CONTINUE
C
C   TAKES LOG OF RATE CONSTANTS

```







C

```
DO 281 I=1,J
W(I)=ALOG(XK(I))
281 CONTINUE
WRITE(6,303)
WRITE(6,302) (W(I),I=1,J)
RETURN
END
```



```

IBMAP BCLEAR
      ENTRY BCLEAR
BCLEAR PZE      **
      TSX      S.PLOC,4
      PZE      S.FBOU,,149
      TRA*     BCLEAR
      END
IBFTC EVALV
C
C
C      THIS ROUTINE INCREMENTS Y(I) AND CHECKS
C      WHETHER IT HAS GONE NEGATIVE OR NOT.
C      IF IT HAS, IT DUMPS
C
C      SUBROUTINE EVALV
      COMMON F,XK,Y,D,X,DL,XK1,XK2,XK3,XK4,XK5,TL,T,XLP,E,
1ERROR,H,DT,KC,N,J,M,KQ,KP,KS,KBLANK,GI,L,LT,LS,
2LR,LQ,LQ,LP,LM,LN,LL,YA,YM,KSTEP,KSTOP,KDUMP,
3KRUN,KDAMP,KNT,W,AR,BR,CR,KRKG,KM,KN,KR,KT,P,U,
4KB,K,R,LA,LB,LC,LD,LE,LF,LG,LH,LI,LJ,LK
      DIMENSION F(100),XK(100),Y(100),D(100),X(10),DL(25),
1XK1(100),XK3(100),XK4(100),XK5(100),KS(10,3),YA(100),
2YM(100),AR(4),BR(4),CR(4),W(100),Z(100),P(100),
3U(100),R(100),KA(98)
      LN=LN+1
20  FORMAT(1X,24H Y(I) IS NEGATIVE I = , I5)
      DO 10 KC=1,N
      Y(KC)= .5*(XK1(KC) +XK5(KC)) +2.*XK4(KC)+Y(KC)
      CALL REREAD
C
C      CALLS KATA PROGRAM IF KT = 2
C
      IF(KT-2) 8,9,9
      9  CALL KATA
      8  CONTINUE
C
C      MAKES SURE THAT Y(I) IS POSITIVE
C
      IF(Y(KC)) 11,10,10
      10 CONTINUE
      RETURN
C
C      DUMPS IF Y(I) IS NEGATIVE
C
      11 WRITE(6,20) I
      CALL RAWD
      RETURN
      END

```





IBFTC CONS

C  
C  
C  
C  
C

THIS SUBROUTINE CALCULATES MASS ACTION VAUES AND  
IS TO BE USED ONLY WITH THE APPROPRIATE PROGRAM

SUBROUTINE CONS

COMMON F,XK,Y,D,X,DL,XK1,XK2,XK3,XK4,XK5,TL,T,XLP,E,  
1ERROR,H,DT,KC,N,J,M,KQ,KP,KS,KBLANK,GI,L,LT,LS,  
2LR,LQ,LO,LP,LM,LN,LL,YA,YM,KSTEP,KSTOP,KDUMP,  
3KRUN,KDAMP,KNT,W,AR,BR,CR,KRKG,KM,KN,KR,KT,P,U,  
4KB,K,R,LA,LB,LC,LD,LE,LF,LG,LH,LI,LJ,LK  
DIMENSION F(100),XK(100),Y(100),D(100),X(10),DL(25),  
1XK1(100),XK3(100),XK4(100),XK5(100),KS(10,3),YA(100),  
2YM(100),AR(4),BR(4),CR(4),W(100),Z(100),P(100),  
3U(100),R(100),KA(98)

LE=LE+1

C

SUBSTRATE PLUS PRODUCT

R(1)=Y(1)+Y(4)

C

ENZYME PLUS COMPLEX

R(2)=Y(2)+Y(3)

RETURN

END



IBFTC REREAD

C  
C  
C  
C

THIS SUBROUTINE INPUTS A NEW VALUE FOR A  
CHEMICAL WHEN IT HAS REACHED A CERTAIN CONCENTRATION

SUBROUTINE REREAD

COMMON F,XK,Y,D,X,DL,XK1,XK2,XK3,XK4,XK5,TL,T,XLP,E,  
1ERROR,H,DT,KC,N,J,M,KQ,KP,KS,KBLANK,GI,L,LT,LS,  
2LR,LQ,LO,LP,LM,LN,LL,YA,YM,KSTEP,KSTOP,KDUMP,  
3KRUN,KDAMP,KNT,W,AR,BR,CR,KRKG,KM,KN,KR,KT,P,U,  
4KB,K,R,LA,LB,LC,LD,LE,LF,LG,LH,LI,LJ,LK  
DIMENSION F(100),XK(100),Y(100),D(100),X(10),DL(25),  
1XK1(100),XK3(100),XK4(100),XK5(100),KS(10,3),YA(100),  
2YM(100),AR(4),BR(4),CR(4),W(100),Z(100),P(100),  
3U(100),R(100),KA(98)

LC=LC+1

IF(Y(KYI).LT.YAMT) GO TO 30

RETURN

30

Y(KYI)=YMT

RETURN

END





IBFTC DERV

C  
C

SUBROUTINE DERV

COMMON F,XK,Y,D,X,DL,XK1,XK2,XK3,XK4,XK5,TL,T,XLP,E,  
1ERROR,H,DT,KC,N,J,M,KQ,KP,KS,KBLANK,GI,L,LT,LS,  
2LR,LQ,LQ,LP,LM,LN,LL,YA,YM,KSTEP,KSTOP,KDUMP,  
3KRUN,KDAMP,KNT,W,AR,BR,CR,KRKG,KM,KN,KR,KT,P,U,  
4KB,K,R,LA,LB,LC,LD,LE,LF,LG,LH,LI,LJ,LK  
DIMENSION F(100),XK(100),Y(100),D(100),X(10),DL(25),  
1XK1(100),XK3(100),XK4(100),XK5(100),KS(10,3),YA(100),  
2YM(100),AR(4),BR(4),CR(4),W(100),Z(100),P(100),  
3U(100),R(100),KA(98)

LS=LS+1

C  
C  
C

SINGLE ENZYME SYSTEM

F(1)=XK(1)\*Y(1)\*Y(2)  
F(2)=XK(2)\*Y(3)  
F(3)=XK(3)\*Y(3)  
F(4)=XK(4)\*Y(4)\*Y(2)

C  
C  
C

SUMMING FLUXES

D(1)=-F(1)+F(2)  
D(2)=-F(1)+F(2)+F(3)-F(4)  
D(3)=-F(2)+F(1)-F(3)+F(4)  
D(4)=+F(3)-F(4)  
RETURN  
END



ENTRY

4 4 1 4 1  
+0.100E+01+0.100E+01+0.000E+00+0.000E+00  
+0.100E+01+0.100E+01+0.100E+01+0.000E+00  
+0.100E-01+0.300E+01+0.100E-03  
+0.100E+01

1 1 S

2 1 E

3 1 C

4 1 P

3 100

3 0 0 0

1 +0.100E-01+0.100E+01

+0.100E+01

END





## APPENDIX

"Simple simulator" program (IBM 7040)



JCB 765003 A.O. OLSON

TIME 8,2000

IBJOB BIO GO

IBFTC MAIN NODCK

COMMON Z(100),Y(100),C(100),F(100),Q(100),AR(4),BR(4),  
1CR(4),DY(100),NPI,NE,DT,TIME,FIN

C CONSTANTS IN THE RUNGE-KUTTE-GILL SUBROUTINE

AR(1)=0.5

AR(2)=0.2929

AR(3)=1.7071

AR(4)=0.1667

BR(1)=2.0

BR(2)=1.0

BR(3)=1.0

BR(4)=2.0

CR(1)=0.5

CR(2)=0.2929

CR(3)=1.7071

CR(4)=0.5

READ (5,11)DT,TIME,FIN

READ (5,12)NPI,NE

READ (5,11)(Z(I),I=1,NPI)

READ (5,11)(C(I),I=1,NE)

WRITE(6,11)DT,TIME,FIN

WRITE(6,12)NPI,NE

WRITE(6,11)(Z(I),I=1,NPI)

WRITE(6,11)(C(I),I=1,NE)

DO 9 I=1,NPI

9 Y(I)=Z(I)

10 CALL RUNGE

TIME=TIME+DT

WRITE(6,14)TIME,(Y(I),I=1,NPI)

WRITE(3,14)TIME,(Y(I),I=1,NPI)

11 FORMAT(8E10.3)

12 FORMAT(1X,3I5)

14 FORMAT(1X,5(5X,E10.3))

IF(TIME.LE.FIN) GO TO 10

16 CALL EXIT

END





```

IBFTC GILL      NCLIST,NODECK
      SUBROUTINE RUNGE
      COMMON Z(100),Y(100),C(100),F(100),Q(100),AR(4),BR(4),
      LCR(4),DY(100),NPI,NE,DT,TIME,FIN
      Q(1)=0.0
      DO 1 J=1,4
      CALL DERIV
      DO 1 I=1,NPI
      DXX=AR(J)*(DY(I)-BR(J)*Q(I))
      Y(I)=Y(I)+DT*DXX
1  Q(I)=Q(I)+3.*DXX-CR(J)*DY(I)
      RETURN
      END

```



```

IBFTC DER      NODECK
      SUBROUTINE DERIV
      COMMON Z(100),Y(100),C(100),F(100),Q(100),AR(4),BR(4),
      1CR(4),DY(100),NPI,NE,DT,TIME,FIN
C      SINGLE ENZYME SYSTEM
      DY(1)=-C(1)*Y(1)*Y(2)+C(2)*Y(3)
      DY(2)=-C(1)*Y(1)*Y(2)+(C(2)+C(3))*Y(3)
      DY(3)=C(1)*Y(1)*Y(2)-C(2)*Y(3)-C(3)*Y(3)
      DY(4)=C(3)*Y(3)
      RETURN
      END

```





```
ENTRY          MAIN
+0.100E-01+0.000E-00+0.100E+02
      4      4      50
+1.000E+00+1.000E-00+0.000E-00+0.000E-00
+1.000E-00+1.000E-00+5.000E-00+0.000E-00
EXECUTE        AUTOPT
PLOT ALL
SIZE 240,129
PLOT DONE
END
```



## APPENDIX

"Multi-Chance" subprogram (for use in the "Regular Simulator" program)



IBFTC DERV      NODECK

C  
C

SUBROUTINE DERV

COMMON F,XK,Y,D,X,DL,XK1,XK2,XK3,XK4,XK5,TL,T,XLP,E,  
1ERROR,H,DT,KC,N,J,M,KQ,KP,KS,KBLANK,GI,L,LT,LS,  
2LR,LQ,LO,LP,LM,LN,LL,YA,YM,KSTEP,KSTOP,KDUMP,  
3KRUN,KDAMP,KNT,W,AR,BR,CR,KRKG,KM,KN,KR,KT,P,U,  
4KB,K,R,LA,LB,LC,LD,LE,LF,LG,LH,LI,LJ,LK  
DIMENSION F(100),XK(100),Y(100),D(100),X(10),DL(25),  
1XK1(100),XK3(100),XK4(100),XK5(100),KS(10,3),YA(100),  
2YM(100),AR(4),BR(4),CR(4),W(100),Z(100),P(100),  
3U(100),R(100),KA(98)  
2,KA(98)

C  
C  
C  
C  
C  
C

MULTI CHANCE MECHANISM OF OXIDATIVE  
PHOSPHORYLATION BASED ON SLATERS  
REPRESENTATION IN BIOLOGICAL OXIDATIONS 14

F(1)=XK(1)\*Y(2)\*Y(3)  
F(2)=XK(2)\*Y(4)\*Y(5)  
F(3)=XK(3)\*Y(4)\*Y(5)\*Y(6)  
F(5)=XK(5)\*Y(9)\*Y(4)  
F(6)=XK(6)\*Y(10)\*Y(11)  
F(7)=XK(7)\*Y(12)\*Y(13)  
F(8)=XK(8)\*Y(14)\*Y(13)  
F(9)=XK(9)\*Y(11)\*Y(15)  
F(10)=XK(10)\*Y(15)\*Y(16)  
F(11)=XK(11)\*Y(17)\*Y(14)  
F(12)=XK(12)\*Y(18)\*Y(19)  
F(13)=XK(13)\*Y(20)\*Y(21)  
F(14)=XK(14)\*Y(22)\*Y(23)  
F(15)=XK(15)\*Y(24)  
F(16)=XK(16)\*Y(17)\*Y(7)  
F(17)=XK(17)\*Y(16)\*Y(1)  
F(18)=XK(18)\*Y(20)\*Y(5)  
F(19)=XK(19)\*Y(2)\*Y(18)  
F(20)=XK(20)\*Y(21)\*Y(7)  
F(21)=XK(21)\*Y(19)\*Y(1)  
F(22)=XK(22)\*Y(24)\*Y(5)  
F(23)=XK(23)\*Y(22)\*Y(2)  
F(24)=XK(24)\*Y(25)

C  
C  
C

SUMMING OF FLUXES

C

AH2

D(1)=-F(1)+F(2)-F(17)+F(16)-F(21)+F(20)

C

B

D(2)=-F(1)+F(2)+F(18)-F(19)+F(22)-F(23)

C

C

D(3)=-F(1)+F(2)-F(4)+F(3)+F(5)

C

A-C

D(4)=-F(2)+F(1)+F(4)-F(5)

C

BH2





```

D(5)=-F(2)+F(1)-F(18)+F(19)-F(22)+F(23)
C PHOSPHATE
D(6)=-F(3)+F(4)
C A
D(7)=-F(4)+F(3)-F(16)+F(17)-F(20)+F(21)
C ATP
D(8)=-F(4)+F(3)
C DNP
D(9)=-F(5)
C SUCCINATE
D(10)=-F(6)+F(7)
C FLAVOPROTEIN
D(11)=-F(6)+F(7)+F(8)-F(9)
C FUMARATE
D(12)=+F(6)-F(7)
C FPH2
D(13)=+F(6)-F(7)-F(8)+F(9)
C Q
D(14)=-F(8)+F(9)-F(11)+F(10)
C QH2
D(15)=-F(9)+F(8)-F(10)+F(11)
C CYTOCHROME B OXIDIZED
D(16)=-F(10)+F(11)+F(16)-F(17)
C CYTOCHROME B REDUCED
D(17)=+F(10)-F(11)-F(16)+F(17)
C CYTOCHROME C1 REDUCED
D(18)=-F(12)+F(13)-F(19)+F(8)
C CYTOCHROME C OXIDIZED
D(19)=-F(12)+F(13)-F(21)+F(20)
C CYTOCHROME C1 OXIDIZED
D(20)=-F(13)+F(12)-F(18)+F(9)
C CYTOCHROME C REDUCED
D(21)=-F(13)+F(12)-F(20)+F(21)
C CYTOCHROME A-A3 REDUCED
D(22)=-F(14)+F(15)-F(23)+F(22)
C OXYGEN
D(23)=-F(14)+F(15)
C CYTOCHROME A-A3 OXIDIZED
D(24)=+F(15)-F(14)-F(22)+F(23)
C MARKER
D(25)=-F(24)
C ADP
D(26)=-F(3)+F(4)
RETURN
END

```



```

simulator" program)

```





IBFTC DERV      NODECK

C  
C

```
SUBROUTINE DERV
1ERROR,H,DT,KC,N,J,M,KQ,KP,KS,KBLANK,GI,L,LT,LS,
2LR,LQ,LO,LP,LM,LN,LL,YA,YM,KSTEP,KSTOP,KDUMP,
3KRUN,KDAMP,KNT,W,AR,BR,CR,KRKG,KM,KN,KR,KT,P,U,
4KB,K,R,LA,LB,LC,LD,LE,LF,LG,LH,LI,LJ,LK
  DIMENSION F(100),XK(100),Y(100),D(100),X(10),DL(25),
1XK1(100),XK3(100),XK4(100),XK5(100),KS(10,3),YA(100),
2YM(100),AR(4),BR(4),CR(4),W(100),Z(100),P(100),
3U(100),R(100),KA(98)
  LS=LS+1
```

C  
C  
C  
C  
C

MITCHELL TYPE OXIDATIVE PHOSPHORYLATION, AS SET DOWN  
BY SLATER

```
F(1)=XK(1)*Y(1)*Y(2)
F(2)=XK(2)*Y(3)*Y(4)*Y(5)
F(3)=XK(3)*Y(4)*Y(6)*Y(7)
F(4)=XK(4)*Y(2)*Y(8)
F(5)=XK(5)*Y(9)*Y(8)
F(6)=XK(6)*Y(6)*Y(10)
F(7)=XK(7)*Y(10)*Y(11)*Y(11)
F(8)=XK(8)*Y(9)*Y(12)*Y(12)*Y(5)*Y(5)
F(9)=XK(9)*Y(13)*Y(13)*Y(13)*Y(14)*Y(14)*Y(14)
F(10)=XK(10)*Y(15)*Y(15)*Y(15)*Y(16)*Y(16)*Y(16)*Y(7)*
1Y(7)*Y(7)
F(11)=XK(11)*Y(5)*Y(5)*Y(5)*Y(16)*Y(16)*Y(16)
F(12)=XK(12)*Y(17)*Y(17)*Y(17)
F(13)=XK(13)*Y(12)*Y(12)*Y(7)*Y(7)*Y(18)
```

C  
C

SUMMING OF FLUXES

```
D(1)=-F(1)+F(2)
D(2)=-F(1)+F(2)-F(4)+F(3)
D(3)=-F(2)+F(1)
D(4)=-F(2)+F(1)-F(3)+F(4)
D(5)=-F(2)+F(1)-F(11)+F(12)-F(8)+F(7)
D(6)=-F(3)+F(4)-F(6)+F(5)
D(7)=-F(3)+F(4)-F(10)+F(9)-F(13)
D(8)=+F(3)-F(4)+F(6)-F(5)
D(9)=-F(5)+F(6)-F(8)+F(7)
D(10)=+F(5)-F(6)+F(8)-F(7)
D(11)=-F(7)+F(8)-F(13)
D(12)=+F(7)-F(8)-F(13)
D(13)=-F(9)+F(10)
D(14)=-F(9)+F(10)
D(15)=+F(9)-F(10)
D(16)=-F(10)+F(9)-F(11)-F(12)
D(17)=-F(12)+F(11)
D(18)=-F(13)
D(19)=+F(13)
RETURN
END
```





**B29868**